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(54) Title: TREATMENT OF CENTRAL NERVOUS SYSTEM DISEASES WITH NITRIC OXIDE OR PEROXYNITRITE SCAVENGERS

(57) Abstract

The process of treating a disease of the central nervous system with an agent from one or more of the following three classes of agents: (1) nitric oxide scavengers, (2) peroxynitrite scavengers, and (3) agents that either interfere with the synthesis of iNOS in the cell or the enzymatic activity of iNOS in the cell.

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TREATMENT OF CENTRAL NERVOUS SYSTEM DISEASES WITH NITRIC OXIDE OR PEROXYNITRITE SCAVENGERS

BACKGROUND OF THE INVENTION

Field of the Invention

The field of the invention is the treatment of diseases of the central nervous system using either a nitric oxide scavenger, a peroxynitrite scavenger, or an agent that interferes with the activity or cellular production of the enzyme, inducible nitric oxide synthase (iNOS).

The overproduction in the body of nitric oxide (NO) and/or peroxynitrite ($ONOO^-$) has been suggested by some to be a contributing factor to diseases of the central nervous system, particular those that are immune-mediated and/or inflammatory.

The enzyme iNOS is responsible for the production of nitric oxide during an immune response. Nitric oxide combines with superoxide (O_2^-) to form peroxynitrite. Those molecular level considerations are relevant to the present inventions.

An extensively used model system to study multiple sclerosis, an example of a disease treated by the present invention, is experimental allergic encephalomyelitis (EAE) in rats and mice. This model was used for experiments described below.

The present invention, in a general aspect, is the process of treating a disease diagnosed as a disease of the central nervous system with an agent from one or more of the following three classes of agents: (1) nitric oxide scavengers, (2) peroxynitrite scavengers, and (3) agents that either interfere with the synthesis of iNOS in the cell or the enzymatic activity of iNOS in the cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Measurements of local NO levels in EAE using spin-trapping and EPR-spectroscopy.

Figure 2. Effect of daily administration of c-PTIO (carboxy-PTIO) on EAS in SWJX-14 mice. EAE was induced in SWJX mice by two subcutaneous immunizations (d.0 and d.7) of 100 mg PLP in CFA over two injection sites. Mice (N=3) were treated beginning on day 5 post-immunization with 2 mg/mouse c-PTIO twice daily i.p. and was continued until day 16 post-immunization (day 0 being the day of first immunization). Mean severity scores were graded as detailed in Table 1.

Figure 3. Effect of daily administration of c-PTIO on NO levels in the brain and spinal cord of SWXJ-14 mice immunized with PLP. SWXJ-14 mice were immunized as described below for **Figure 1.** Beginning 4 days post-immunization, mice (n=7) were treated with 2 mg c-PTIO given i.p. until 16 days post-immunization. Mean severity scores were graded as detailed in Table. 1. Mean nitric oxide levels in brain and spinal cord were semi-quantitated using spin-trapping with DETC and EPR spectroscopy at 17 days post-immunization as described

elsewhere [2,10]. (For brain or spinal cord, results are represented as follows: Left hand column: untreated. Center column: treated with c-PTIO. Right hand column: no EAE control).

Figure 4. Effect of D609 on the development of EAE in SWXJ-14 mice. SWXJ-mice were immunized with PLP as detailed for **Figure 2**. Mice (n=2) were treated i.p. with 1 mg/mouse D609 from day 5 through day 14 post-immunization. Mean severity scores were graded as detailed in Table 1.

Figure 5. D609 inhibits nitrite production by activated A549 cells in vitro. The accumulation of nitrite over time was measured in the presence and absence of D609. Human A549 cells were activated with IL-1β (100 units/ml), γIFN (500 units/ml and TNFα (10 ng/ml), and the accumulation of nitrite over time was measured using the Griess reaction [15]. The concentration of nitrite was calculated using a standard solution of nitrite in culture media.

Figure 6. Effects of PTIO on clinical severity of EAE in Lewis rats.

Figure 7. Comparison of PTIO and carboxy-PTIO on EAE in SJL mice. For each day, the left hand column represent results for PTIO-treated mice, the center column shows results for c-PTIO-treated mice, and the right hand column shows results for untreated mice.

Figure 8. Mean levels of nitric oxide in EAE immunized SJL mice 18 days post-immunization.

Figure 9. Effect of carboxy-PTIO on EAE in SWXJ-14 mice.

Figure 10. Mean levels of nitric oxide in EAE-induced SWXJ-14 mice. (For the results with the brain, and the results for the spinal cord, the left hand column represent results with untreated mice, the right hand column represents results with c-PTIO-treated mice.)

Figure 11. Effect of carboxy-PTIO on EAE in SWXJ-14 mice.

Figure 12. Effect of the administration of various doses of uric acid on EAE in SWXJ-14 mice. EAE was induced in SWXJ-14 mice by two subcutaneous immunizations (d.0 and 7) of 1900 mg PLP in CFA (complete Freund's adjuvant) over two injection sites. Mice (n=5) were treated once daily, beginning on day 5 post-immunization, with the indicated doses of uric acid. Mean severity scores were graded as detailed in Table 1.

Figure 13. Effect of the administration of uric acid on brain and spinal cord NO levels and the clinical severity of EAE in SWXJ-14 mice. SWXJ-14 mice were immunized with PLP in CFA as described for **Figure 12.** and treated once daily with the indicated doses of uric acid i.p. At day 16 post-immunization the animals were euthanized and NO levels in brain and spinal cord assessed as detailed for **Figure 3.** Results are for individual mice. Each central white column represents the spinal cord NO level. The black column to the left of the white column represents the clinical severity score. The black column to the right of a white column represents the brain NO level.

Figure 14. Comparison of the survival of SWXJ-14 mice with EAE treated with PTIO and uric acid. SWXJ-mice were immunized with PLP in CFA as described for **Figure 12.** and

treated once daily with a 2 mg dose of uric acid or PTIO i.p. from day 5 to day 13, followed by two daily doses on day 14 and three daily doses there afterward.

Figure 15. Cumulative clinical score in SJL-PLJ mice immunized with MBP in CFA and treated with 10 mg of uric acid twice daily either ip or po. Groups of 10 female SJL-PLJ (PL-SJL) mice were immunized s.c. with 100 μg guinea pig MBP in CFA. Six days after immunization twice daily treatment with 10 mg per dose of uric acid i.p. (black, filled squares) or p.o. (white, unfilled squares was commenced. Clinical signs were recorded twice daily according to the scale summarized in **Table 3**. The average clinical signs are shown with moribund and deceased animals given a score of 7. The average score of untreated mice is also shown (black, filled triangles).

Figure 16. Percent survival of SJL-PLJ mice immunized with MBP in CFA and treated with 10 mg of uric acid twice daily either ip or po. The percent survival of mice immunized with MBP and treated i.p. (black, filled squares) or p.o. (white, unfilled squares) with uric acid, 10 mg twice daily, or left untreated (black, filled triangles) is shown.

Figure 17. Percent survival of SJL-PLJ mice immunized with MBP in CFA and treated with 10 mg of uric acid once, twice, or four times daily starting at 5 or 24 days after immunization. Groups of 10 SJL-PLJ were immunized with MBP in CFA and pertussis toxin on day 0. Starting 5 days later, groups were treated with 10 mg uric acid i.p. once (white, unfilled circles), twice (black, filled circles), or four

(black, filled squares) times daily or with saline (black, filled triangles). One group received 4 doses per day starting at 24 days after immunization (white, unfilled diamonds) (denoted by the arrow).

Figure 18. Effect of uric acid treatment from day 12 post-immunization with MBP on the development on clinical signs of EAE in SJL-PLJ mice. Groups of 10 female SJL-PLJ mice were immunized with MBP in CFA. Twelve days afterwards mice that remained healthy were either treated with uric acid (black, filled circles) twice daily with 10 mg per dose for 7 days or left untreated (white, unfilled circles). Clinical score was assessed twice daily as detailed in **Figure 15**.

Figure 19. Effect of uric acid treatment from day 12 post-immunization with MBP on the survival of SJL-PLJ mice without symptoms when treatment was started. Groups of 10 female SJL-PLJ mice were immunized with MBP in CFA. Twelve days afterwards mice that remained healthy were either treated with uric acid (black, filled circles) twice daily with 10 mg per dose for 7 days or left untreated (white, unfilled circles).

Figure 20. Effect of uric acid treatment from day 12 post-immunization with MBP on clinical signs of EAE in SJL-PLJ mice. Groups of 10 female SJL-PLJ mice were immunized with CFA. Twelve days afterwards mice with clinical signs were split into two groups, either treated with uric acid (black, filled squares) twice daily with 10 mg per dose for 7 days or left untreated (white, unfilled squares). Clinical score was assessed twice daily as detailed in **Figure 15**.

Figure 21. Effect of uric acid treatment from day 12 post-immunization with MBP on the survival of SJL-PLJ mice with EAE. Groups of 10 female SJL-PLJ mice were immunized with MBP in CFA. Twelve days afterwards mice with clinical signs were separated into groups that were either treated with uric acid (black, filled squares) twice daily at 10 mg per dose for 7 days or left untreated (white, unfilled squares).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

GLOSSARY AND DEFINITIONS

"PLP" is proteolytic protein from the myelin sheath, specifically PLP 139-151 [8] (the "8" in brackets refers to reference 8, below).

"MBP" is myelin basic protein, an autoantigen from the myelin sheath of nerves, a target of much damage in multiple sclerosis.

"PMBP" is a peptide with an amino sequence found in MBP.

"MS" is multiple sclerosis.

"PTIO" is 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide.

"Carboxy-PTIO" is 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide.

"Uric Acid" is 2,6,8-trihydroxypurine.

"D609" is tricyclodecan-9-yl-xanthogenate. D609 is believed to block the activation of PC-PLC by blocking PC-PLC activation.

"AIDS" is acquired immune deficiency syndrome.

"PC-PLC" is phosphatidylcholine-specific phospholipase C. PC-PLC is thought to be involved in a signal transduction pathway that leads, via the transcription factor NF-κB to the activation of iNOS.

A "scavenger of NO" is a compound that by reacting with NO eliminates the ability of NO to act as a free radical. Similarly, a "scavenger of peroxynitrite" is a compound that by reacting with peroxynitrite eliminates the ability of peroxynitrite to act as a free radical.

ASPECTS OF THE INVENTION

In a general aspect, the present invention is a process of treating a disease of the central nervous system of a mammal (such as a human), the process comprising administering to the mammal a pharmacologically effective dose of one or more agents that are either (1) a nitric oxide scavenger, (2) a peroxynitrite scavenger, or (3) an agent that either interferes with the synthesis of iNOS in the cell or the enzymatic activity of iNOS in the cell.

An agent that inhibits the synthesis or enzymatic activity of iNOS is referred to here as an anti-iNOS in the cell.

A pharmacologically effective dose is one that slows or prevents the progression of the disease being treated. It is preferable that the slowing or prevention not be accompanied by a toxic effect that offsets the medical value of slowing the progression of the targeted disease of the central nervous system.

In a particular embodiment of the treatment process, a pharmacologically active dose of a nitric oxide scavenger is administered, regardless of whether or not a peroxynitrite scavenger or an anti-iNOS agent is administered.

In another embodiment of the treatment process, a pharmacologically active dose of a peroxynitrite scavenger is administered, regardless of wether or not a nitric oxide scavenger or an anti-iNOS agent is administered.

In another embodiment of the treatment process, a pharmacologically active dose of an anti-iNOS agent is administered, regardless of whether or not a nitric oxide scavenger or a peroxynitrite scavenger is administered.

It is preferable to administer two or three independently acting agents rather than a single agent. Therefore one preferred embodiment of the process is the administration of both a nitric oxide scavenger and a peroxynitrite scavenger. Another preferred embodiment is the administration of both a nitric oxide scavenger and an anti-iNOS agent. Similarly, another preferred embodiment is the administration of both a peroxynitrite scavenger and an anti-iNOS agent. A fourth most preferred embodiment is the administration of nitric oxide scavenger, a peroxynitrite scavenger, and an anti-iNOS agent.

The diseases of the central nervous system that are targets for this invention include those that, in addition to being a disease of the central nervous system, may affect or involve parts of the body other than the central nervous system. As a result, diseases that are targets for this invention include:

multiple sclerosis;

Alzheimer's disease;

AIDS with general symptoms;

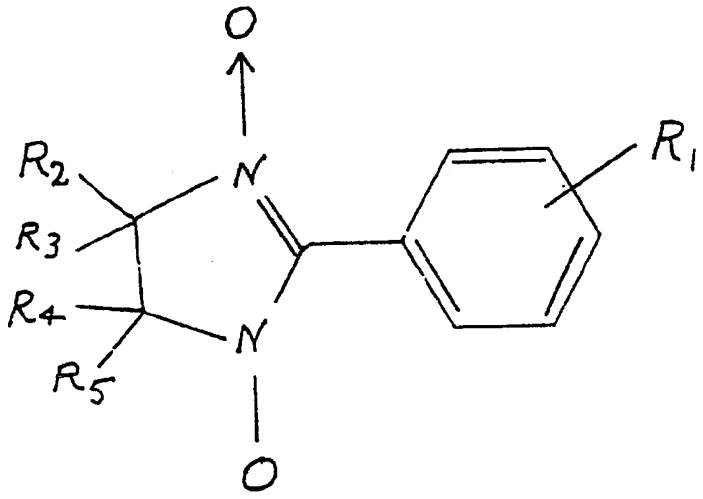
amyotrophic lateral sclerosis;

cerebral malaria;

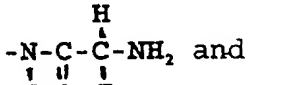
Pick's disease; and

any form of virus-induced encephalitis (e.g., herpes encephalitis).

Preferred nitric oxide scavengers are

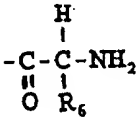


wherein R_1 is -H, $-CH_3$ $-CH_2OH$, $-NH_2$, -SH, -OH, $-SO_3$, or



and

wherein



corresponds to amino acid whose carboxyl group has been modified by its reaction with an $-NH_2$ group (the amino acid preferably selected from the group, serine, threonine, asparagine, glutamine, lysine, arginine, histidine, glutamic acid, aspartic acid, cysteine, glycine, proline, alanine,

isoleucine, leucine, methionine, phenylalanine, tryptophan, valine and tyrosine) and corresponding derivative, that R_2, R_3, R_4 and R_5, are independently selected (but preferably the same) from the group, methyl, ethyl, propyl, isopropyl and *n*-butyl.

Preferred nitric oxide scavengers are:

2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide;

2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; and

2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide.

The production of those nitric oxide scavengers is discussed in U.S. patent 5,464,857., and J.H. Osiecki et al., J. Am. Chem. Soc. vol. 90, pp 1078-1079 (1968) which J Am. Chem. Soc. is article is hereby incorporated by reference

Preferred peroxynitrite scavengers are, in preferred embodiments of the invention, related to uric acid (2,6,8-trihydroxypurine): They are selected from the group consisting of uric acid and compounds that are uric acid mono- or di-substitued, a substituent being at the 1-, 3-, 7-, or 9- position of uric acid, a substituent being selected from the group consisting of $-CH_3$, $-C_2H_5$, $-OH$, $-CH_2OH$, $-NH_2$, $-SH$, and $-SO_3$. Highly preferred compounds are selected from the group consisting of 2,6,8-trihydroxypurine, 1-methyluric acid, 3-methyluric acid, 7-methyluric acid, 9-methyluric acid, 1,3-dimethyluric acid, 1,7-dimethyluric acid, 1,9-dimethyluric acid, 3,7-dimethyluric acid, 3,9-dimethyluric acid, 7,9-dimethyluric acid. Additional preferred peroxynitrite

scavengers are dihydrorhodamine, ascorbic acid, methionine, cysteine, glutathione, cysteine methyl ether, penicillamine and, generally compounds that contain a thiol group (especially glutathione or cysteine). Uric acid is also considered to be an hydroxyl radical scavenger.

In a related aspect of the invention, a compound that is directly or indirectly metabolized in a person to uric acid is added to achieve an increase in the amount of uric acid, so as to treat multiple sclerosis or other disease named in this patent application. Highly preferred for such a treatment is xanthine (the immediate precursor of uric acid) or the purines, hypoxanthine, guanine, and adenine). Recommended human dosages for the preferred compounds are those given elsewhere herein for uric acid.

A preferred anti-iNOS agent is tricyclodecan-9-yl-xanthogenate.

A test for interference with nitric oxide synthase activity is an arginine-to-citrulline assay as follows: Activated (with or without interfering agent) or control Raw 264.7 (mouse) cells are detached from culture vessels with trypsin, washed twice with phosphate-buffered saline (PBS), counted, and resuspended in five volumes of lysis buffer (50 mM Tris, pH 7.5, 1 mM dithiothreitol and protease inhibitor mixture Complete [Boehringer Mannheim, Indianapolis, IN]). Cells are lysed by three freeze-thaw cycles and lysate is cleared by centrifugation at 15,000 x g for 15 min. The reaction is initiated by addition of lysis buffer containing (final concentrations) 1 mM NADPH, 10 μM (6R)-5,6,7,8

tetrahydro L biopterin HCl, 100 μM CaCl, 50 nM calmodulin, 10 μM FAD, and 1 μCi ^{3}H-L-arginine. The mixture is incubated for 60 min at 37°C, and a 150 μl aliquot is loaded onto a cation exchange column (AG 50 W-Xg, Bio-Rad, Hercules, CA) to resolve the citrulline from the arginine, then eluted with water. The fractions are collected and the radioactivity present is quantified by liquid scintillography. The general NOS inhibitor methyl L-arginine and the type 11 NOS inhibitor trifluoperazine can be used to confirm that the NO assay is working properly.

Uric acid reduces the amount of dihydrorhodamine converted. Compounds that per mole of compound are at least half as effective as the same molar amount of uric acid in the assay, when the assay is done under conditions where the reduction effect of uric acid increases with its concentration, are preferred.

The identification of a peroxynitrite scavenger is done as follows: Peroxynitrite formation is measured in vitro by the peroxynitrite-mediated oxidative conversion (in the presence or absence of scavenger) of dihydrorhodamine 123 (DHR 123) to the fluorescent rhodamine 123 [Kooy, N.W., Royall, J.A., Ischiropoulos, H., and Beckman, J.S. (1994) Peroxynitrite-mediated oxidation of dihydrorhodamine 123. Free Radic. Biol. Med., 16:149-156, (which is hereby incorporated by reference), and Szabo, C., Salzman, A.L., and Ischiropoulos, H. (1995) Peroxynitrite-mediatedoxidation of dihydrorhodamine 123 occurs in early stages of endotoxic and hemorrhagic shock and ischemia-reperfusion injury.

F.E.B.S., 372:229-232, (which is also hereby incorporated by reference)]. Other potential oxidants of dihydrorhodamine, including peroxide, superoxide, and nitric oxide, have negligible effects on the compound at physiological concentrations (Szabo, et al. (1995)). Peroxynitrite produced by release from the compound SIN (3-morpholinosydnonimine.HCL) is detected in this assay. Dihydrorhodamine 123 is added to phosphate-buffered saline (PBS) containing SIN at 5 μM for up to 60 minutes. Rhodamine 123 produces in the PBS is then measured in a fluorimeter at an excitation wavelenght of 485 nm and an emission wavelenght of 530 nm. Peroxynitrite content is quantified based on a standard curve obtained with different amounts of SIN.

The identification of a NO scavenger is done as follows: NO production in culture is estimated by the accumulation of nitrite, (in the presence or absence of scavenger) the stable product produced by the breakdown of NO, which can be measured by the Griess reaction. [Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., and Tannenbaum, S.R. (1982) Analysis of Nitrate, Nitrate and [^{15}N] Nitrate in Biological Fluids. Analytical Biochem., 126:131-138, which is hereby incorporated by reference]. Culture supernatants (200 μl) are mixed with 100 μl of Griess reagent (0.05% N-(1-napthyl)ethylenediamine dihydrochloride and 0.5% sulfanilamide in 2.5% phosphoric acid) and allowed to react for 5 min. A standard curve is prepared using defined concentrations of sodium nitrite solution in culture medium. Optical densities are read at 550 nm. NO can also be measured using a spin-

trapping agent and cryogenic electron paramagnetic resonance (EPR) spectroscopy using diethylamine-NONOate, an NO donor to make a calibration curve [Kubrina, L.N., Caldwell, W.S., Mordvintcev, P.I., Malenkova, I.V., and Vanin, A.F. (1992) Biochem. Biophys.Acta. 1099:233-237], (which is hereby incorporated with reference.), and Hooper, D.C., Ohnishi, T.S., Kean, R., Numagami, Y., Dietzschold, B., and Koprowski, H. (1995) Local nitric oxide production in viral and autoimmune diseases of the central nervous system. P.N.A.S.,92:5312-5316,(which is hereby incorporated with reference)]. This technique, which has been used to detect NO production in vivo, involves administering a spin-trapping agent diethyldithiocarbamate (DETC, 500 mg/kg) and a ferrous sulfate/sodium citrate mixture (20 mg/kg and 100 mg/kg respectively), snap freezing the tissue at -80°C, and measuring the spectra characteristic of NO in a spectrometer with a liquid nitrogen flow system at 140K [Kubrina, et al. (1992)]. The conversion of PTIO to PTI, resulting from its reaction with NO, is measured with this approach.

MODES OF ADMINISTRATION

Modes of administration of the various therapeutic agents used in the invention are exemplified in the Examples below. However, the agents can be delivered by any of a variety of routes including: by injecting (e.g., subcutaneous, intramuscular, intravenous, intraarterial, intraperitoneal), by continuous intravenous infusion, orally (e.g., tablet, pill, liquid medicine), by implanted osmotic pumps (e.g., Alza Corp.), by suppository or by aerosol spray.

DOSAGE

The extensive data in the Examples provide a useful starting point for calculating human dosage requirements.

For uric acid, and uric acid derivatives (such as methyl-substitued or dimethyl-substituted uric acid) the preferred dose range is normally between 1 mg/kg body weight/dose and 1 g/kg body weight/dose; one to three doses per day is normally preferred. However, if the uric acid or uric derivative is administered locally (e.g. at the site of inflammation) the dose may be as low as 10 μg/kg body weight.

For the other preferred peroxynitrite scavengers, the preferred dose ranges and local site requirements are the same as those to those for uric acid.

Preferred dosage regimens for PTIO and its derivatives in humans are those for uric acid. Preferred human D609 dosage is about 50 mg/kg body weight once daily.

Pharmaceutically acceptable salts

Pharmaceutically acceptable salts of the agents may be used, as may reagents such as appropriate esters that are modified by the body's enzymes to liberate the agent of interest.

Examples

Induction of EAE in rats by adoptive transfer of MBP-specific T cells or in SJL or SWXJ-14 mice by immunization with MPB or PLP 139-151, a peptide derived from MBP [8] results in variable disease. We have scored the clinical systems of EAE as tabulated below.

Table 1. Severity scores and symptoms of Experimental allergic Encephalomyelitis

Score	Clinical Systems
1	piloerection, tail weakness
2	tail paralysis
3	hind limb weakness/paralysis
4	hind and forelimb paralysis
5	moribund

As we have previously described, the severity of clinical symptoms of EAE as well as viral encephalopathies that are positive immune-mediated evidently correlates with NO production in the CNS [14]. We have determined that the site of major NO production varies between different EAE models. **Figure 1** shows the results of NO measurement in the brains and spinal cords of rats with adoptive transfer EAE versus SWXJ-14 mice with immunization-elicited EAE. The adoptive transfer of MBP-specific T cells in Lewis rats causes NO production which is largely limited to the spinal cord while immunization of SWXJ-14 mice with PLP 139-151 results in the elaboration of high levels of NO in both spinal cord and brain.

In **Figure 1**, EAE was elicited in 11 week old female Lewis rats by the adoptive transfer of 20 x 10^6 cells of the MBP-specific line 5HGBP.G5 activated by stimulation with MBP and syngeneic APC (antigen presenting cells)48 hours previously and NO levels measured 5 days after transfer. In SWXJ-14 mice, EAE was triggered by two subcutaneous immunizations (d.0 and 7) with 100 μg PLP in CFA over two injection sites and NO

levels were measured 18 days later. The results shown represent the mean values obtained from a minimum of 4 animals. Peak severity of the symptoms of EAE ranged from 2-3 for the rats and 4-5 for the mice. NO was semi-quantitated using spin trapping with DETC and EPR spectroscopy as described previously [10A].

Our initial studies in rats with EAE elicited by the adoptive transfer of MBP-specific T cells and in SJL mice immunized with MBP showed that i.p. administration of appearance of clinical symptoms of EAE as well reduced levels of NO detected in spinal cord by spin-trapping and EPR spectroscopy **(Table 2).**

Table 2. Effects of PTIO and carboxy-PTIO treatment on adoptive transfer EAE in Lewis rats and on immunization-elicited EAE in SJL mice.

EAE model[1]	Treatment[2]	NO-spinal[3] cord (mM)	Clinical[4] severity
Rat-adoptive transfer	none	12.3	3
	PTIO	undetectable	0.8
SJL mouse - immunization	none	2.4	1.5
	PTIO	1.6	0.8
	c-PTIO	1.4	0.7

[1]EAE was induced in Lewis rats by adoptive transfer of MBP-specific T cells and in SJL mice by immunization as detailed in the legend to **Figure 1**.

[2]Beginning on day 2, rats were treated daily with 100 mg/kg PTIO given i.p. Commencing ten days post-immunization, groups of mice (n=6) were treated once daily with 2 mg of either PTIO or carboxy-PTIO given i.p.

[3]Spinal cord NO levels ere determine by spin-trapping and EPR spectroscopy a previously described [10A]. Measurements were taken on day 5 post-immunization for rats and day 18 post-immunization for mice.

[4]Mean severity scores were graded at the time of sacrifice as detailed in **Table 1**.

In contrast to Lewis rats an SJL mice which often exhibit only mild symptoms and recover from EAE, SWXJ-14 mice immunized against myelin undergo a progressive, often fatal form of EAE. Clinical symptoms manifest as an ascending paralysis approximately 13 days after immunization and the disease rapidly progresses to its fatal endpoint by days 16-20. As is apparent form the results represented in **Figure 2**, daily treatment of SWXJ-14 mice with two doses of 100 mg/kg carboxy-PTIO, commencing 4 days following immunization with PLP, delays the onset and reduces the severity of the clinical symptoms of EAE. NO levels in the brains and spinal cords of animals treated with a single daily dose of PTIO were semi-quantitated by spin trapping and EPR spectroscopy on day 17 after immunization, one day after treatment was terminated. Interestingly, the mice treated with carboxy-PTIO had low levels of NO in brain tissue compared to untreated PLP-immunized controls while spinal cord NO had already reached significant levels (**Figure 3**). These were, however, somewhat less than the spinal cord NO levels detected in the surviving untreated animals. We expect that the removal of NO by PTIO should not necessarily interfere with the induction of the response responsible for NO production and that as the

supplied PTIO is used up, NO levels should rapidly return to control values.

We have employed the human lung carcinoma cell line A549 (ATCC) and, as for comparison, the mouse monocyte-macrophage cell line RAW 264.7 (ATCC) as producers of NO. Treatment of RAW 264.7 cells with 1 αg/ml of LPS (lipopolysaccharide)or A549 cells with IL-1β (100 μg/ml), γIFN (500 units/ml), and TNFα (10 ng/ml) stimulates the production of NO which can be detected by the accumulation of nitrite in culture supernatants. Using these methods of stimulation, RAW cells produced up to 100 uM of nitrite during 24 hours of culture while the A549 cells generally produced roughly 10-fold less nitrite in our experiments. As shown in **Figure 5** inclusion of D609 (50 ug/ml)in the culture medium completely inhibited nitrite production by the A549 cells. Three other PLC inhibitors also blocked nitrite accumulation in cultures of both mouse and human cells (data not shown).

Rats were injected i.v. with 20 x 10^6 MBP-specific CD4 T cells activated in vitro. Two days later the animals were treated with a single dose of either PTIO (20 mg) or a mixture of PTIO (20 mg), indomethacin (1 mg), and allopurinol (13 mg). Treatment was continue on a daily basis until day 4 and animals were sacrificed for NO measurement the following day. As shown in **Figure 6**, rats treated with PTIO alone exhibited little or no symptoms of disease, while treatment with the mix had little effect.

Since the PTIO is not water soluble (it can be administered in a lipid formulation) and therefore somewhat

problematic in use, especially at the doses employed, we next compared the effectiveness of PTIO and a water soluble derivative, carboxy-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] in other models of EAE to establish whether or not these compounds have a similar therapeutic effect. **Figure 7** shows the results of this comparison. SJL mice are immunized subcutaneously with PMB in adjuvant to induce EAE, from which they often recover. Commencing ten days post-immunization, groups of mice (n=6) were treated once daily with 2 mg of either PTIO or carboxy-PTIO intraperitoneally and observed for clinical signs of EAE which were graded as detailed in Table 1. Both PTIO and carboxy-PTIO treated mice exhibited delayed onset of disease and somewhat lessened severity by comparison with untreated animals. At 18 days post-infection, NO determinations were performed in several mice from each group. The NO levels detected were lower in the spinal cords of mice treated groups with PTIO and carboxy-PTIO (**Figure 8**). The minimal increase in brain NO caused by the induction of EAE was also inhibited by carboxy-PTIO (**Figure 8**).

In contrast to SJL mice, SWX mice immunized with PLP undergo a progressive, often fatal form of EAE. Clinical symptoms manifest as an ascending paralysis approximately 13 days after immunization and the disease rapidly progresses to its fatal endpoint by days 16-20. As is apparent from the results represented in **Figure 9**, treatment of SWX mice with a single daily dose of 100 mg/kg mg carboxy-PTIO, commencing 4 days following immunization with PLP, delays the onset and

reduces the severity of the clinical symptoms of EAE. Interestingly, when NO levels were semiquantitated on day 17 after immunization, carboxy-PTIO treatment had evidently prevented the induction of NO in brain while spinal cord NO had already, one day after treatment had concluded, reached significant levels (**Figure 10**). These did however remain somewhat less than the spinal cord NO levels detected in the surviving untreated animals. **Figure 11** shows that treatment of PLP-immunized SWXJ-14 mice with two daily doses of approximately 2 mg carboxy-PTIO dramatically improves the clinical course of EAE by comparison with a single daily dose (**Figure 9**). It could be that the amount of carboxy-PTIO we administered, especially considering the short in vivo half life of the compound, was only sufficient to delay the onset of the EAE in the SWXJ-14 mouse. Nevertheless, carboxy-PTIO administration limited NO production to the spinal cord and had a clear therapeutic effect in these animals despite their exaggerated EAE.

The fact that the onset of the clinical symptoms of EAE can be delayed and their severity diminished by treatment with NO scavengers confirms that NO is involved in the pathogenesis of this MS-related autoimmune process. Since EAE mediated by T cell transfer, which circumvents the activation of the antigen-specific elements of immunity involved in the disease process, can also be successfully treated with carboxy-PTIO, we conclude that NO is intimately involved in the end or effector stage of the disease. Thus, PTIO treatment should be effective regardless of the nature of the stimuli and cells

involved in the production of NO. A significant feature of this aspect of the action of PTIO is that blocking the effects of NO by removing the molecule may not influence the immune mechanisms leading to its production and, more importantly, the regulatory processes that may eventually control the disease process. Thus, we would not expect that treatment with PTIO would interfere with whatever mechanism is involved in the periods of spontaneous remission often seen in patients with MS.

The biological half-life of carboxy-PTIO is estimated to be less than five minutes in the blood when injected i.v. or i.p. into either rats or mice. Approximately 75% of the PTIO administered to mice can be accounted for in excretions within 4 hours [17]. Nevertheless, the clinical symptoms of animals subjected to the stimuli that induce EAE and treated with PTIO are clearly alleviated for at least 24 hours between doses. This suggests that although the action of PTIO in vivo may be very short-lived, the effects of its activity are longer lasting. It is possible that the removal of a significant quantity of NO may reduce the amount of tissue damage to a level that the animal can cope with. However, we believe that it is possible that the transient reduction of NO accumulation in an inflammatory response may have more wide ranging effects for instance through the disturbance of regulatory circuits effecting the production of NO or one of the other related substances involved in immunopathology.

Another possibility, suggested by the difference between spinal cord and brain in the "recovery" of NO levels in EAE

following withdrawal of PTIO treatment, is that PTIO treatment may have a protective effect by maintaining the integrity of the blood brain barrier. Through its effects on the circulation, NO may play a role in facilitating access of MBP specific T cells to the brain which could be inhibited by PTIO. It is also conceivable that a PTIO derivative formed by its interaction with NO also has some protective effect. Whatever the case, we conclude that the NO scavenger PTIO has a strong therapeutic effect in EAE without notable side-effects which may be considered possible due to the normal actions of NO in other physiological systems. The fact that the onset of the clinical symptoms of EAE can be delayed and their severity diminished by reducing brain and spinal cord NO levels through treatment with NO scavengers, tends to substantiate the hypothesis that NO may be involved in the pathogenesis of this autoimmune process.

Also noteworthy is the finding that PTIO treatment is effective when commenced after the disease process has been initiated. PTIO treatment is effective when started 4-5 days after immunization with myelin antigens and EAE mediated by T cell transfer, which circumvents the activation of the antigen-specific elements of immunity involved in the disease process, can also be successfully treated with carboxy-PTIO. In addition, the rapid onset of symptoms of EAE, and spinal cord production, when PTIO treatment is withdrawn also argues that both inflammatory myelin-specific T cells and macrophage are present and may be active in the spinal cords of the treated animals. We therefore conclude that the action of

PTIO, and therefore NO, is intimately involved in the end or effector stage of the disease. Thus, PTIO treatment should be effective regardless of the nature of the stimuli and cells involved in the production of NO. A significant feature of this aspect of the action of PTIO is that blocking the effects of NO by removing the molecule may not influence the immune mechanisms leading to its production and, more importantly, the regulatory processes that may eventually control the disease process. Thus, we would expect that treatment with PTIO would not interfere with whatever mechanism is involved in the periods of spontaneous remission often seen in patients with MS.

NO is not universally considered to be a molecule with significant in vivo toxicity and peroxynitrite is thought to be the more toxic molecule produced by activated inflammatory cells resulting from the interaction of NO and superoxide. We would henceforth expect that removal of NO would reduce peroxynitrite formation. To determine whether NO or peroxynitrite is more likely to be ultimately responsible for clinical disease in EAE we have performed a series of experiments using uric acid, a known scavenger of peroxynitrite, to treat EAE induced in SWXJ-14 mice by immunization with PLP. As demonstrated in **Figure 12**, uric acid treatment commencing at day 5 post-immunization delays the onset and severity of the clinical symptoms of EAE in these animals in a dose-dependent fashion. The majority of the mice treated with a high dose of uric acid remained healthy throughout the length of the experiment.

At 16 days after immunization, several mice from each groups were euthanized so that NO levels in their brains and spinal cords could be measured using spin-trapping and EPR spectroscopy. As can be seen in **Figure 13**, significant levels of NO were detected in spinal cord and brain of mice treated with uric acid that failed to show symptoms of EAE. We therefore conclude that NO itself may not be entirely responsible, directly, for clinical disease in EAE and that peroxynitrite has a major role.

To more directly compare the action of PTIO and uric acid in EAE we have administered each of these agents in parallel to PLP-immunized SWXJ-14 mice. In these experiments the mice were immunized with PLP and treated with increasing doses of PTIO or uric acid beginning at day 5 after immunization. **Figure 14** shows the results of a representative experiment.

While the untreated controls died or were euthanized due to severe disease by day 18, 40 and 80% of the mice treated with PTIO and uric acid, respectively, were still alive several days later and a number were actually beginning to show some improvement in the clinical signs of their disease when the experiment was terminated. This suggests the possibility that treatment with scavengers of NO and, particularly, peroxynitrite may allow SWXJ-14 mice suffering from EAE to undergo remission, which is an exceedingly rare event in this model.

In the SJL-PLJ mouse EAE model, intra-peritoneal (i.p.) administration of uric acid has proven to be most effective in suppressing the appearance of the clinical signs of the

disease. Oral administration of the same amount of uric acid was found to be somewhat less effective (**Figures 15, 16**). This is likely to be due to the lesser amounts of uric acid which are taken up after feeding. There is considerable evidence that the oral route of administration should be highly effective for man. Mice, unlike man, metabolize uric acid one step further to allantoin prior to excretion in the urine. In mice, ingested uric acid may therefore be metabolized to allantoin, which is ineffective as a scavenger of peroxynitrite [Whiteman and Halliwell, 1996], before reaching the serum or lymphatics in large enough quantities to mediate its effect. In man, uric acid is the end stage of purine metabolism and, through a balanced specific excretion/re-uptake mechanism, is excreted as much in urine. In man, uric acid is evidently readily taken up through the digestive system. Dietary uric acid and uric acid precursors are well known to effect changes in serum uric acid in man as documented by many studies of the effect of diet on hyperuricemia. Since uric acid is a end-stage metabolite in man, it may also be argued that pharmacological intervention to raise uric acid levels may be as effective as administration of exogenous uric acid, or a useful, possibly even necessary in some people, adjunct to uric acid administration. A number of pharmacologically active compounds which raise serum uric acid levels in man have been identified.

The results presented in **Figures 15** and **16** show that 2 doses of 10 mg each of uric acid delays but does not prevent

EAE as expected if this dose of uric acid is overcome by a combination of peroxynitrite production and metabolic breakdown. If this is the case, a more comprehensive dose regimen would be expected to have a greater therapeutic effect. The results shown in **Figure 17** demonstrates that 4 daily doses of 10 mg uric acid are in fact more protective in EAE than a single or two daily doses. In addition, the results in **Figure 17** show that 4 daily doses of 10 mg uric acid have a significant therapeutic effect even if started late after immunization.

Table 3. GRADING SYSTEM FOR CLINICAL ASSESSMENT OF EAE

0 - normal mouse

1 - piloerection, tail weakness

2 - tail paralysis

3 - tail paralysis and hind limb weakness (forelimb weakness without hind limb or tail effect*)

4 - tail and partial hind limb paralysis (forelimb paralysis without hind limb or tail effect*)

5 - tail and complete hind limb paralysis (forelimb paralysis without hind limb or tail effect*)

6 - general paralysis

7 - moribund

*rare

(**TABLE 3** is used for **Figures 15,18** and **20**)

The effect of uric acid treatment late in the acute stage of EAE in SJL-PLJ mice was investigated. Treatment of mice with nitric oxide and peroxynitrite scavengers is highly effective

at suppressing the onset of immunization-induced EAE even if commenced 5 days after immunization, at a time when it is likely that the pathogenic immune response is well underway. To determine the effectiveness of uric acid treatment in mice at later stages of EAE we began treatment with uric acid 12 days after immunization when one mouse had already died and approximately 50% had significant clinical signs of EAE. As shown in **Figures 18** and **19**, 7 days of i.p. uric acid treatment of MBP-immunized mice delayed the appearence of clinical signs and death in mice that showed no clinical signs when the treatment started. **Figures 20** and **21** describe the effects of uric acid treatment of mice with clinical signs of EAE at 12 days post-immunization with MBP.

The results of these experiments demonstrate that uric acid treatment, in a dose dependent manner, can not only forestall the appearance of clinical signs of EAE but also can reverse the progression of the disease. Chronic administration of uric acid appears to be required to maintain the therapeutic effect.

A distinct but allied approach towards the inhibition of toxic NO-related effects in chronic neurological diseases is to interfere with the induction of the enzyme responsible for the formation for NO, iNOS. Towards this end we have employed the xanthogenate D-609 which inhibits the induction of iNOS by blocking the activation of phosphatidylcholine-specific phospholipase C (PC-PLC). PC-PLC activation is proximal step in the signal transduction pathway that leads to the activation of iNOS [16]. As can be seen from the results

expressed in **Figure 4**, daily administration of 1 mg D609 delays the onset of the clinical symptoms of EAE elicited by pMBP immunization of SWXJ-14 mice.

Separate experiments involving gel-shift assays, not described in detail here, showed that D609 (50 μg/ml) inhibits the transcriptional activation of the iNOS gene in human A549 cells activated with 1 μg/ml of LPS (bacterial lipopolysaccharide) and mouse RAW 264.7 cells activated with a mixture of IL-1β (100 units/ml), γIFN (500 units/ml) and TNFα (10 ng/ml), also that the activation of the transcription factor NF-KB in those human and mouse cells.

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* * *

CLAIMS

1. A process of treating a disease of the central nervous system of a mammal, such as a human, which process comprises administering to the mammal a pharmacologically effective dose of one or more agents that are either (1) a nitric oxide scavenger, (2) a peroxynitrite scavenger, or (3) an anti-iNOS agent, an anti-iNOS agent being an agent that either interferes with the synthesis of inducible nitric acid synthase in the mammal or the enzymatic activity of inducible nitric acid synthase.

2. A process of Claim 1 wherein the mammal is a human.

3. A process of Claim 2 wherein a peroxynitrite scavenger is administered.

4. A process of Claim 3 wherein the peroxynitrite scavenger is 2,6,8-trihydroxypurine.

5. A process of claim 3 wherein the disease of the central nervous system is either multiple sclerosis, Alzheimer's disease, AIDS with general symptoms, amyotrophic lateral sclerosis, cerebral malaria, Picks disease, or a virus-induced encephalitis.

6. A process of claim 4 wherein the disease of the central nervous system is either multiple sclerosis, Alzheimer's disease, AIDS with general symptoms, amyotrophic

lateral sclerosis, cerebral malaria, Picks disease, or a virus-induced encephalitis.

7. A process of Claim 3 wherein the disease is multiple sclerosis.

8. A process of Claim 4 wherein the disease is multiple sclerosis.

9. A process of Claim 3 wherein a peroxynitrite scavenger and a nitric oxide scavenger is administered.

10. A process of Claim 3 wherein a peroxynitrite scavenger and an anti-iNOS agent are administered.

11. A process of Claim 9 wherein a nitric oxide scavenger, a peroxynitrite scavenger and an anti-iNOS agent are administered.

12. A process of Claim 2 wherein a nitric oxide scavenger is administered.

13. A process of Claim 12 wherein the nitric oxide scavenger is selected from the group consisting of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, and

-35-

-4,4,5,5-tetramethylimidazoline-1-

laim 13 wherein the nitric oxide
a the group consisting of
ıylimidazoline-1-oxyl-3-oxide, and
5-tetramethylimidazoline-1-oxyl-3-

aim 13 wherein the disease of the
is either multiple sclerosis,
with general symptoms, amyotrophic
ral malaria, Picks disease, or a
s.

Claim 15 wherein the disease is

laim 12 wherein the human is not
ue of a fall in blood pressure.

laim 16 wherein the human is not
ue of a fall in blood pressure.

Claim 12 wherein a nitric oxide
)S agent are administered.

lateral sclerosis,
virus-induced encepha

7. A process of
sclerosis.

8. A process of C
sclerosis.

9. A process of
scavenger and a nitric oxi

10. A process of C
scavenger and an anti-iNOS

11. A process of Cla
scavenger, a peroxynitrite sc
are administered.

12. A process of Claim
scavenger is administered.

13. A process of Claim 1
scavenger is selected from the gr
2-phenyl-4,4,5,5-tetramethylimida
2-(4-carboxyphenyl)-4,4,5,5-tetram
oxide, and

20. A process of Claim 2 wherein an anti-iNOS agent is administered.

21. A process of Claim 20 wherein the disease of the central nervous system is either multiple sclerosis, Alzheimer's disease, AIDS with general symptoms, amyotrophic lateral sclerosis, cerebral malaria, Picks disease, or a virus-induced encephalitis.

22. A process of Claim 21 wherein the anti-iNOS agent is tricyclodecan-9-yl-xanthogenate.

23. A process of Claim 22 wherein the disease is multiple sclerosis.

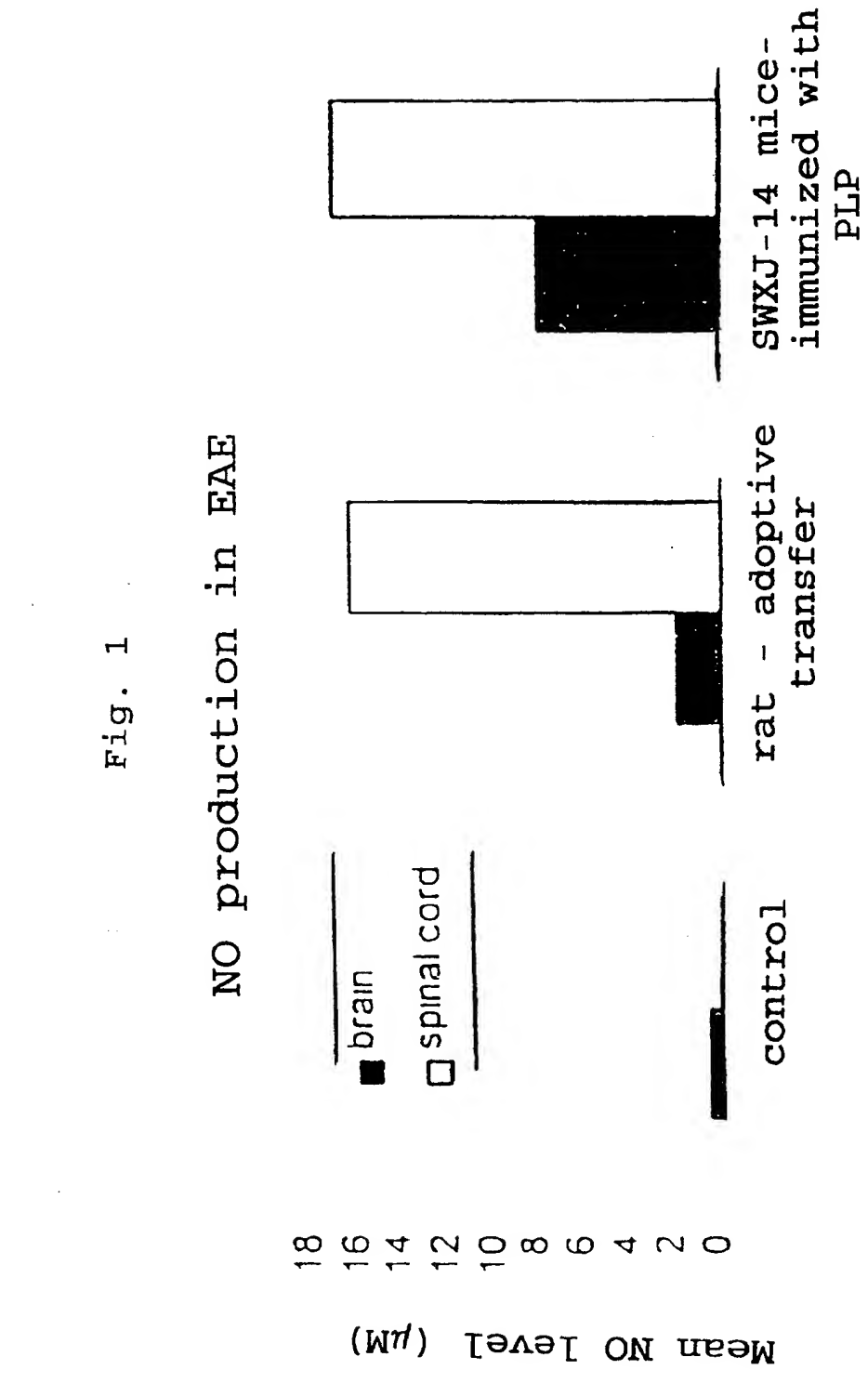

NO production in EAE
Mean NO level (μM)
18
16
14
12
10
8
6
4
2
0
brain
spinal cord
control
rat - adoptive transfer
SWXJ-14 mice-immunized with PLP
EAE model

Fig. 1

Fig. 2

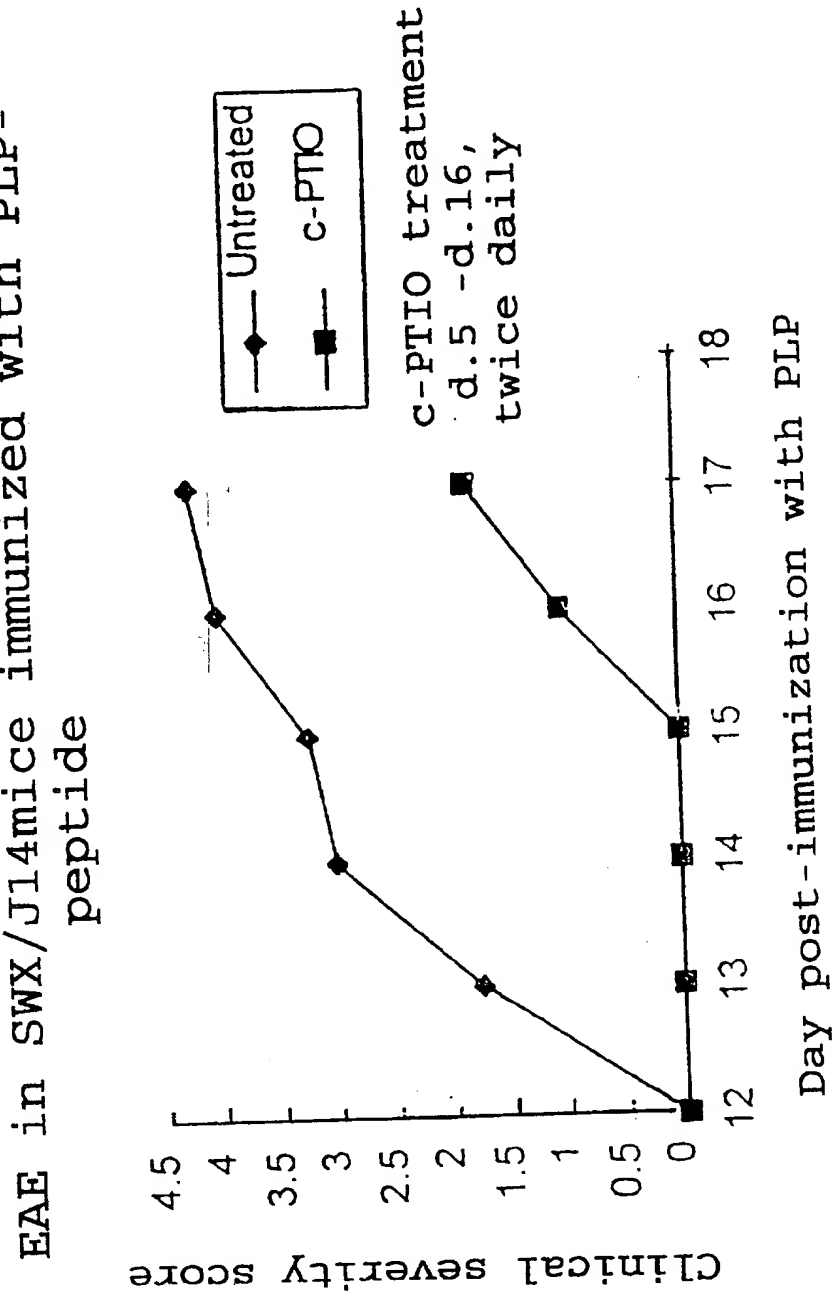

EAE in SWX/J14mice immunized with PLP-peptide
Untreated
c-PTIO
c-PTIO treatment d.5 -d.16, twice daily
Clinical severity score
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
12
13
14
15
16
17
18
Day post-immunization with PLP

Fig. 3

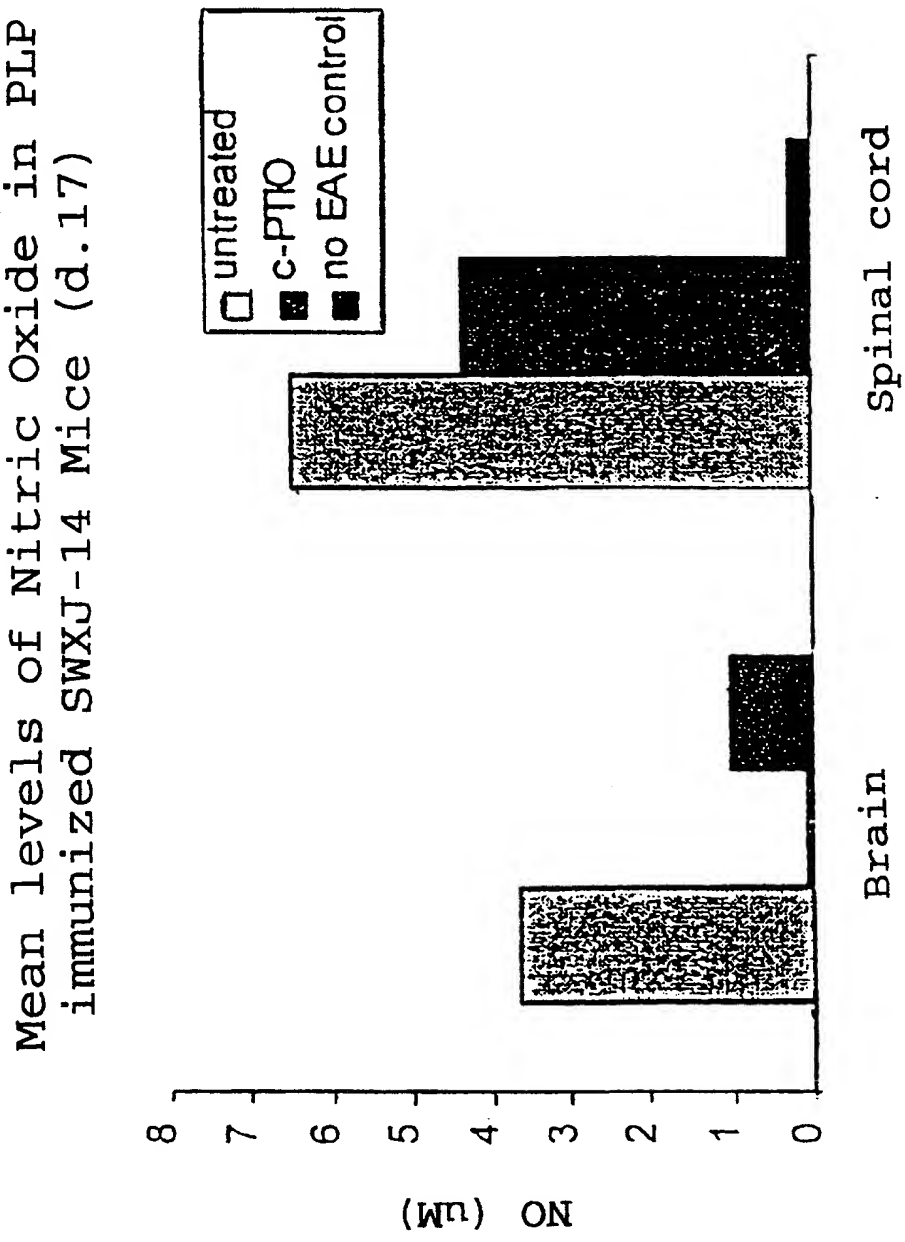

Mean levels of Nitric Oxide in PLP immunized SWXJ-14 Mice (d.17)
untreated
c-PTIO
no EAE control
NO (uM)
0
1
2
3
4
5
6
7
8
Brain
Spinal cord

Fig. 4

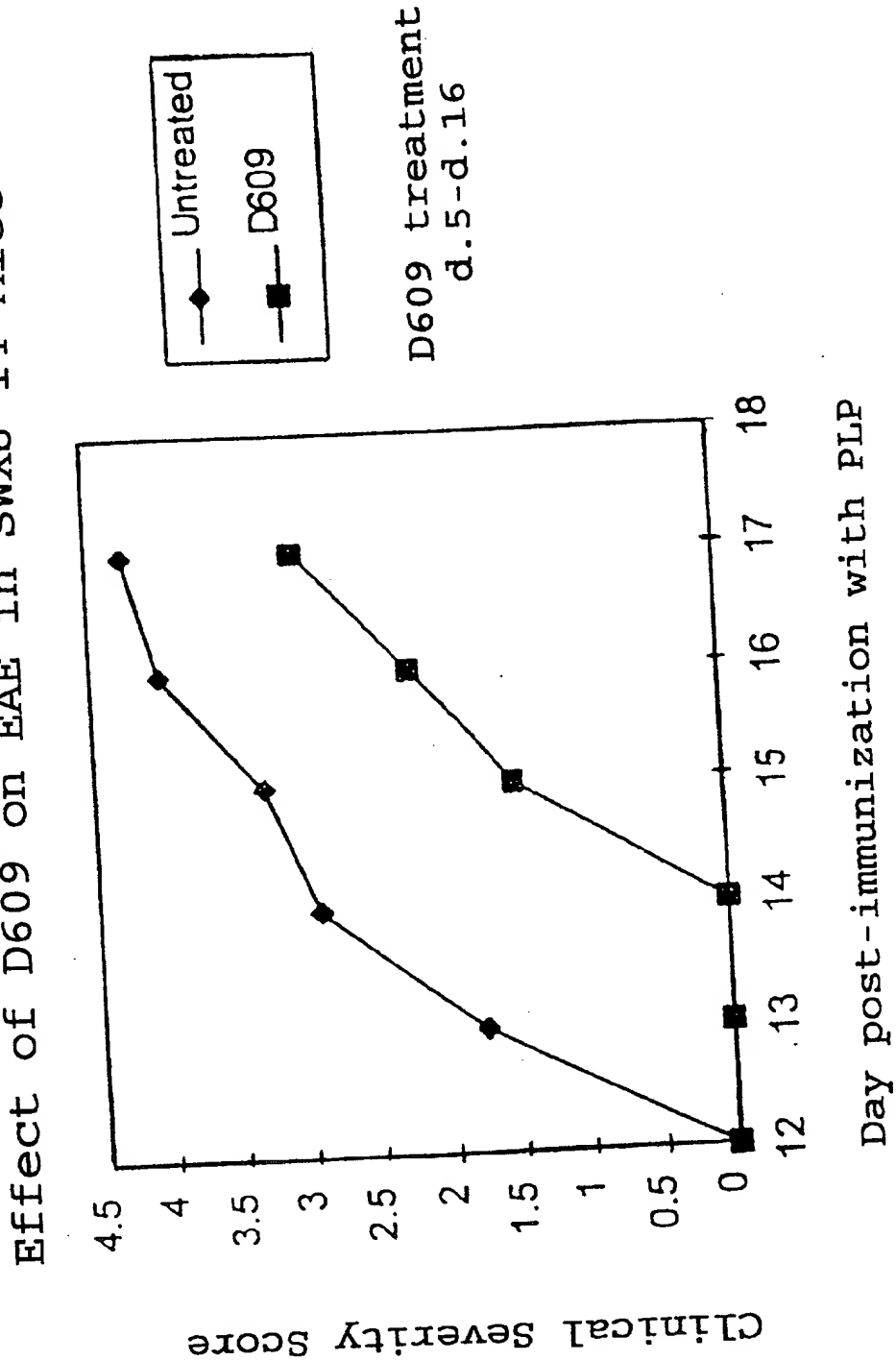

Effect of D609 on EAE in SWXJ-14 Mice
Untreated
D609
D609 treatment
d.5-d.16
Clinical Severity Score
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
12
13
14
15
16
17
18
Day post-immunization with PLP

Fig. 5

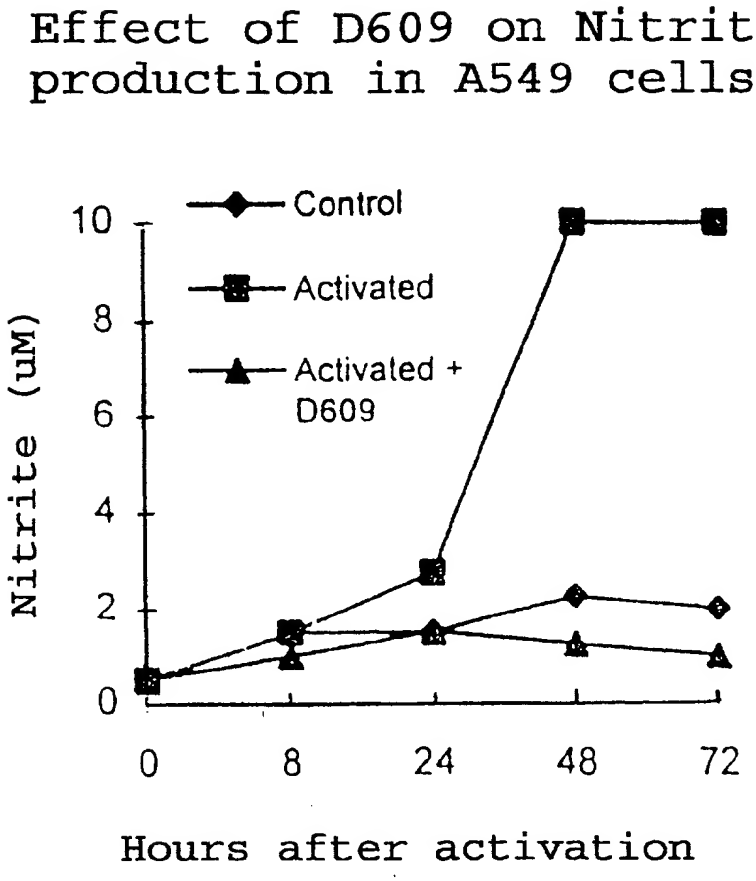

Effect of D609 on Nitrite production in A549 cells
Control
Activated
Activated + D609
Nitrite (uM)
10
8
6
4
2
0
0
8
24
48
72
Hours after activation

Fig. 6

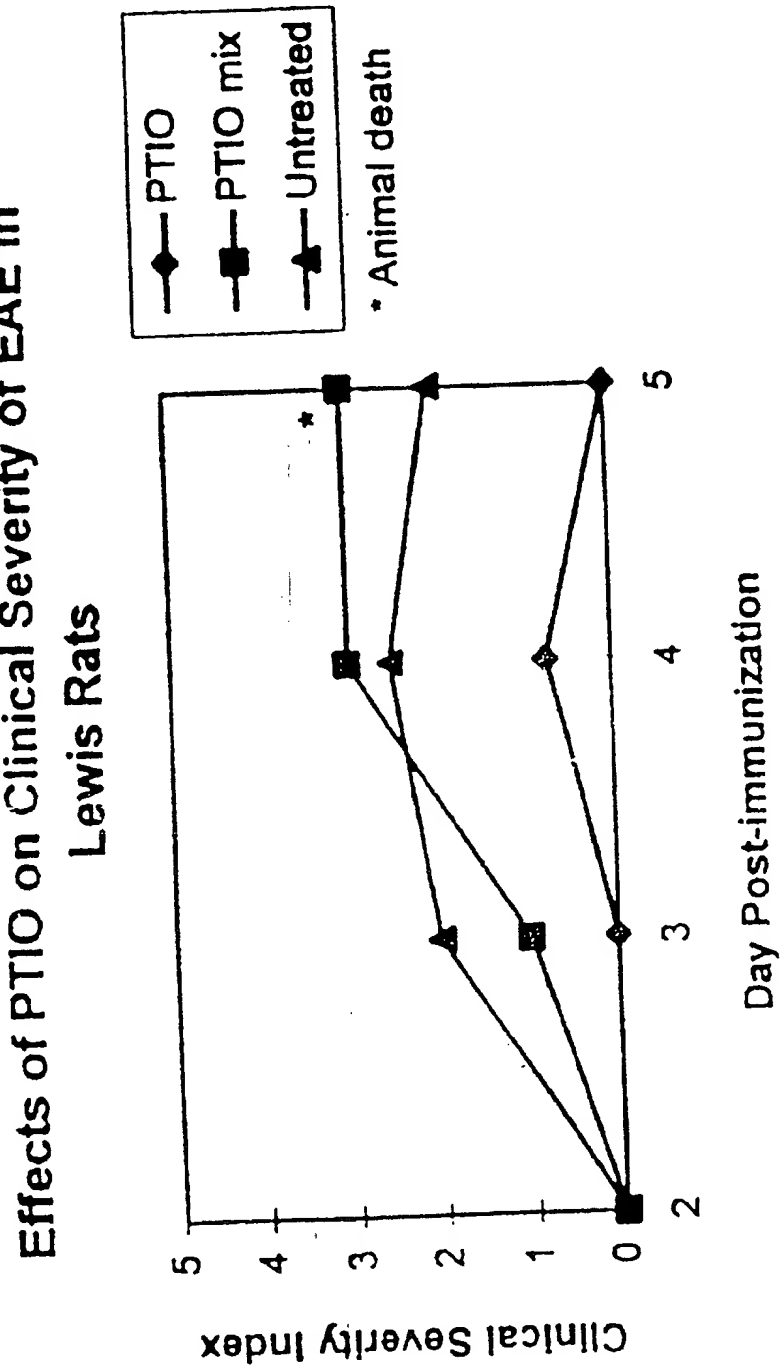

Effects of PTIO on Clinical Severity of EAE in Lewis Rats
PTIO
PTIO mix
Untreated
* Animal death
Clinical Severity Index
0
1
2
3
4
5
2
3
4
5
Day Post-immunization

Fig. 7

Comparision of PTIO and carboxy-PTIO on EAE in SJL mice

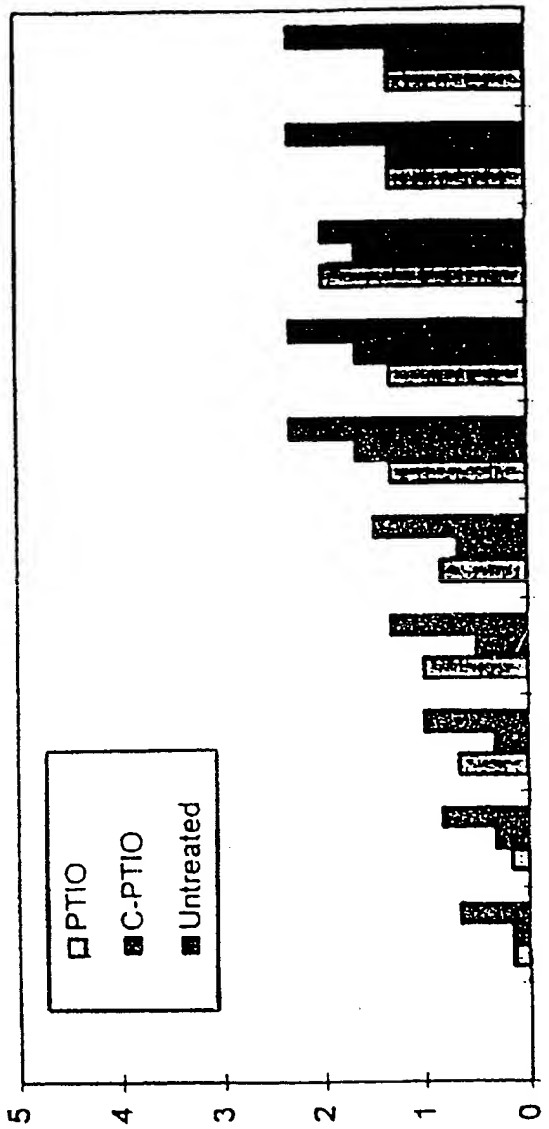


Fig. 8

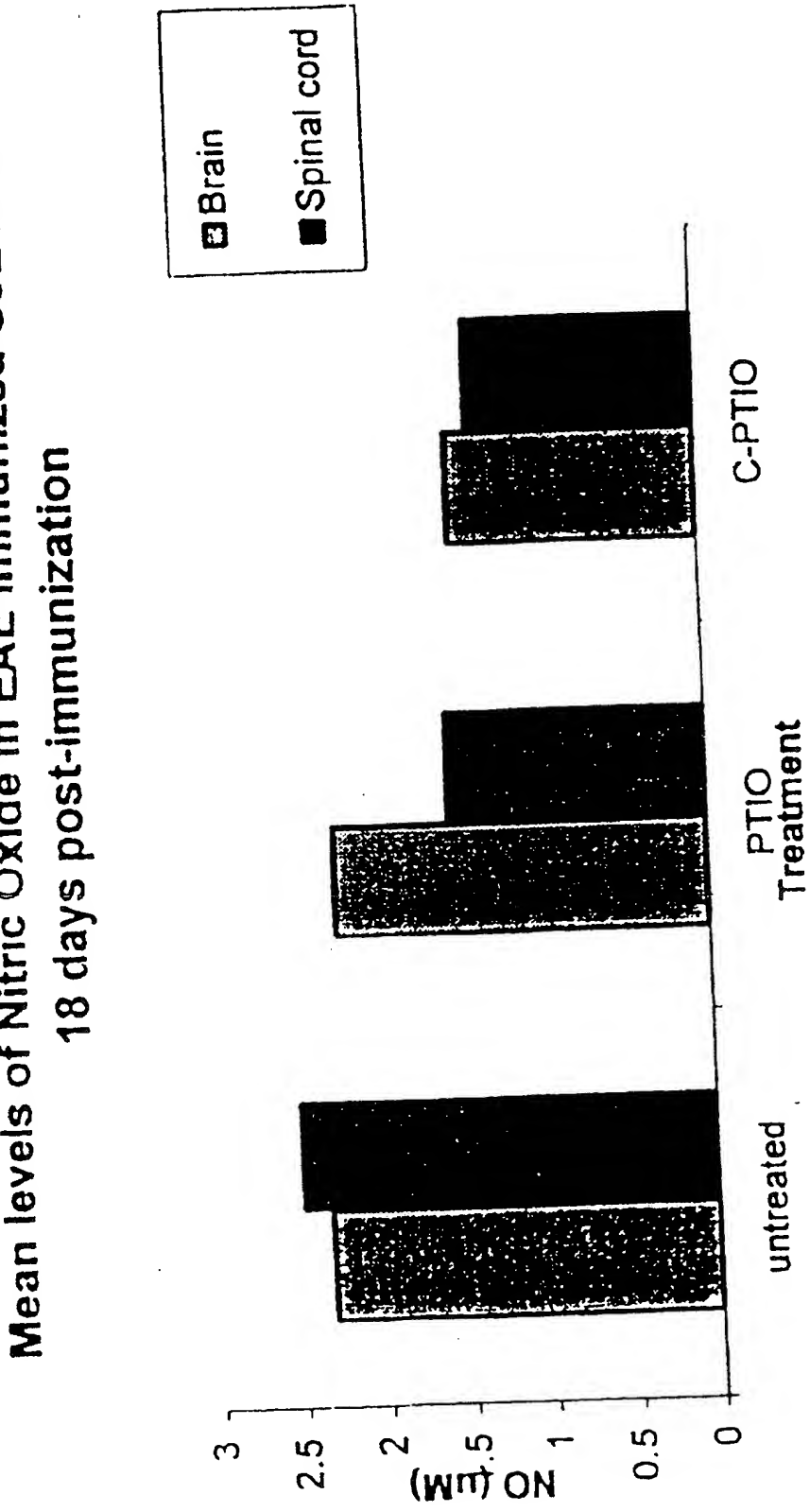

Mean levels of Nitric Oxide in EAE immunized SJL mice
18 days post-immunization
Brain
Spinal cord
NO (μM)
3
2.5
2
1.5
1
0.5
0
untreated
PTIO
C-PTIO
Treatment

Fig. 9

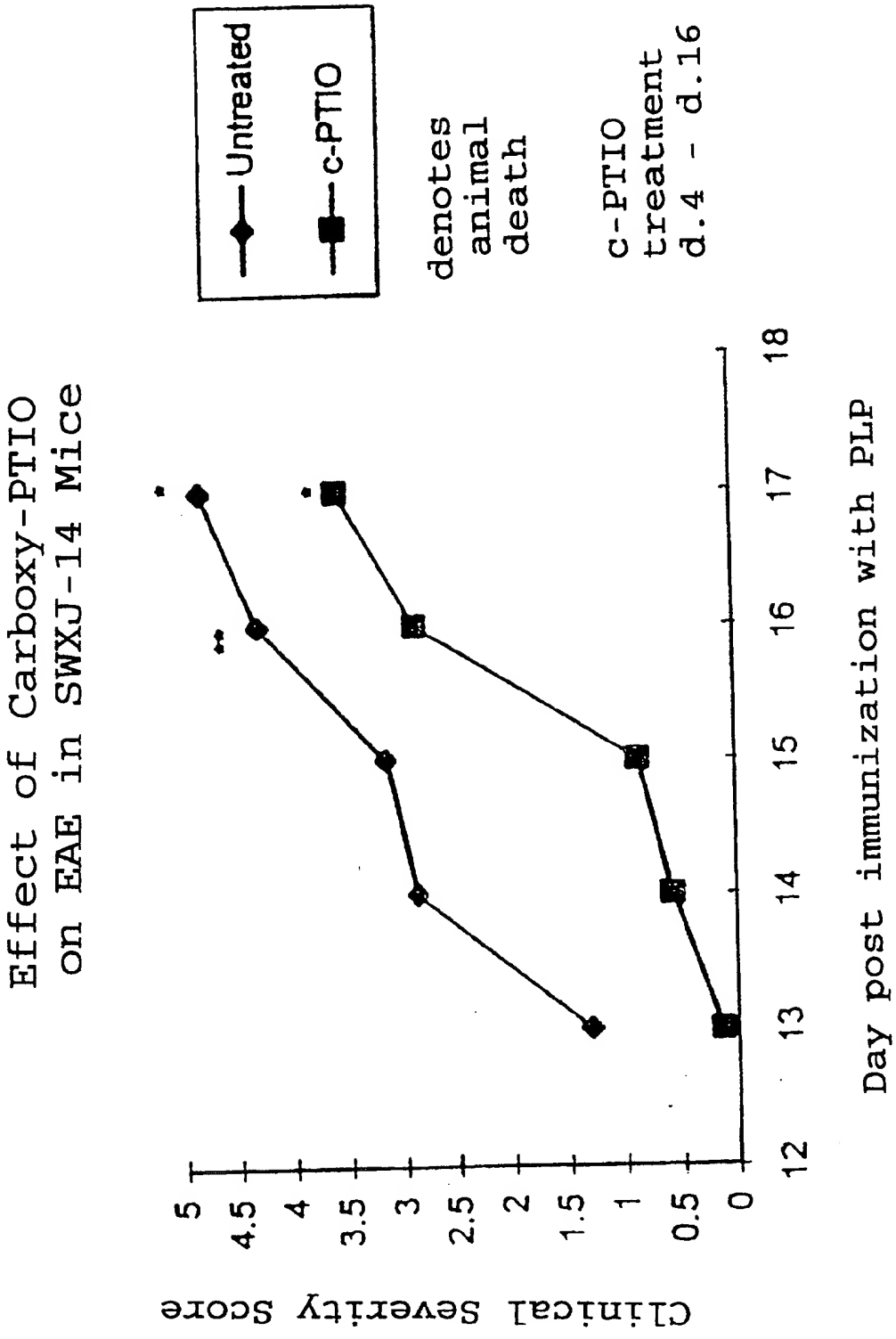

Effect of Carboxy-PTIO
on EAE in SWXJ-14 Mice
Untreated
c-PTIO
denotes
animal
death
c-PTIO
treatment
d.4 - d.16
Clinical Severity Score
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
12
13
14
15
16
17
18
Day post immunization with PLP

Fig. 10

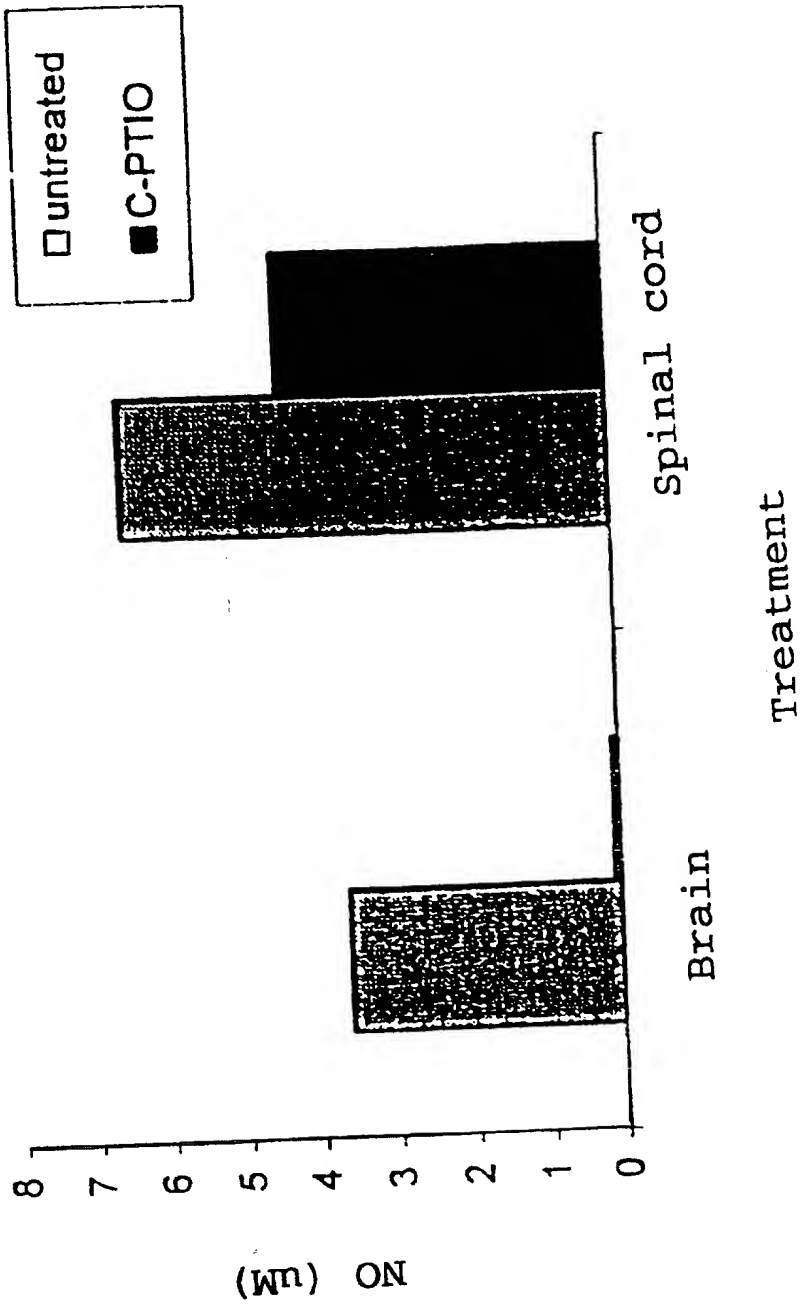

Mean levels of Nitric Oxide in
EAE-induce SWXJ-14 Mice
untreated
C-PTIO
NO (uM)
0
1
2
3
4
5
6
7
8
Brain
Spinal cord
Treatment

Fig. 11

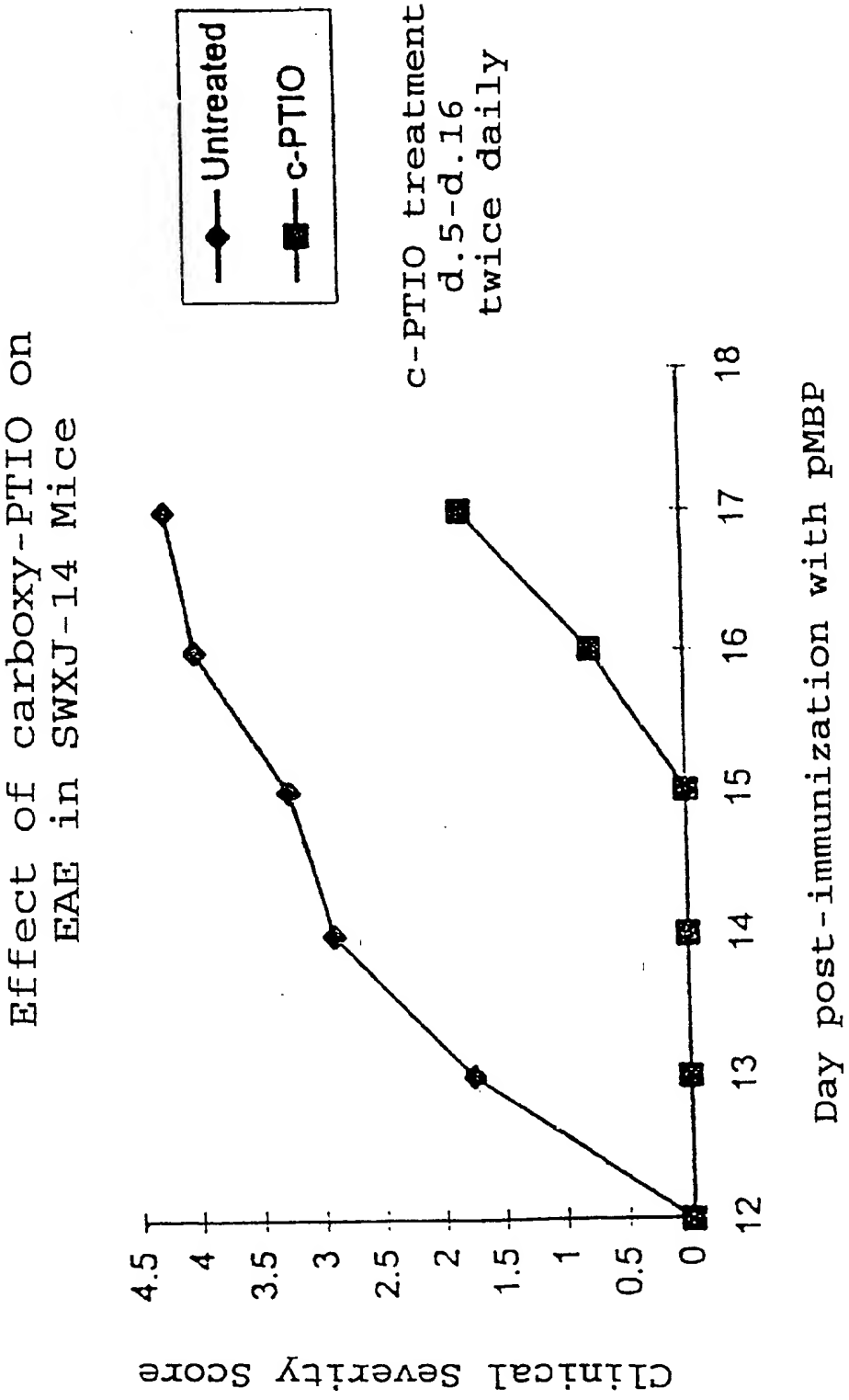

Effect of carboxy-PTIO on
EAE in SWXJ-14 Mice
Untreated
c-PTIO
c-PTIO treatment
d.5-d.16
twice daily
Clinical severity score
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
12
13
14
15
16
17
18
Day post-immunization with pMBP

Fig. 12

Effect of Uric acid on EAE in SWXJ-14 Mice

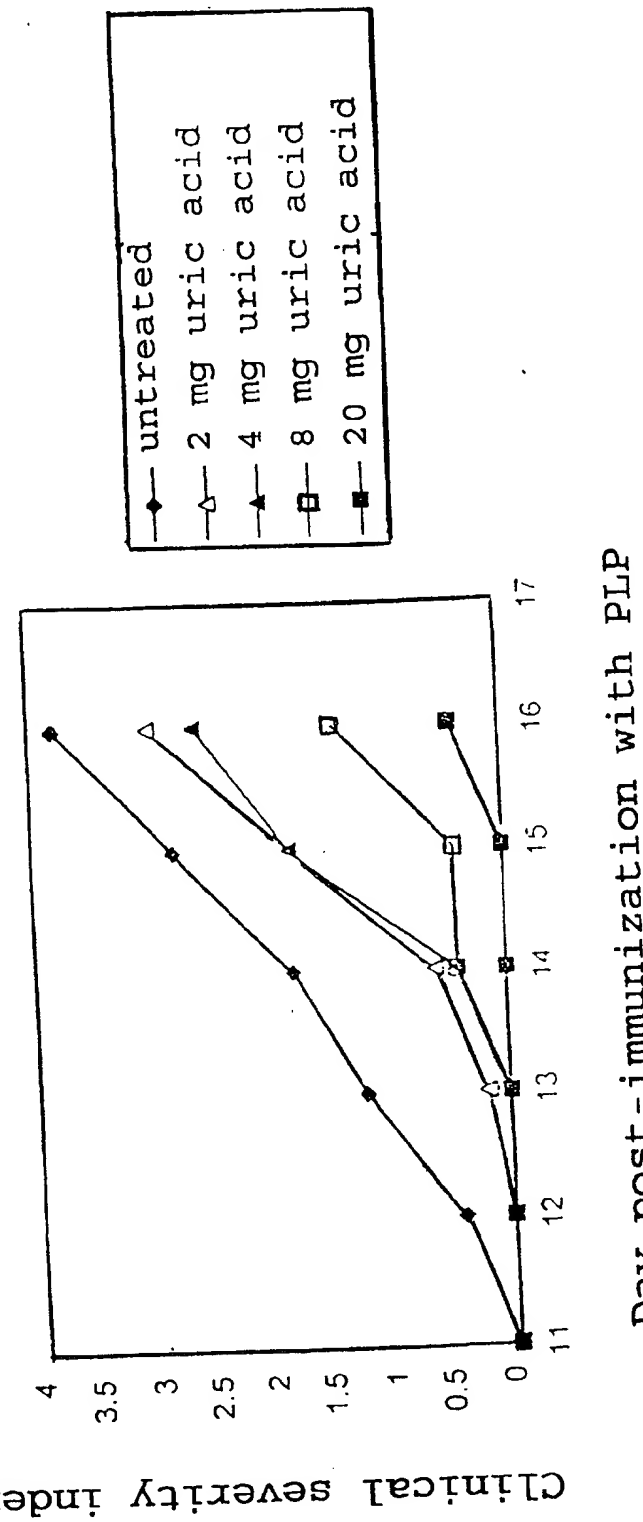


Fig. 13

NO levels and clinical severity of EAE in SWXJ-14 mice treated with uric acid

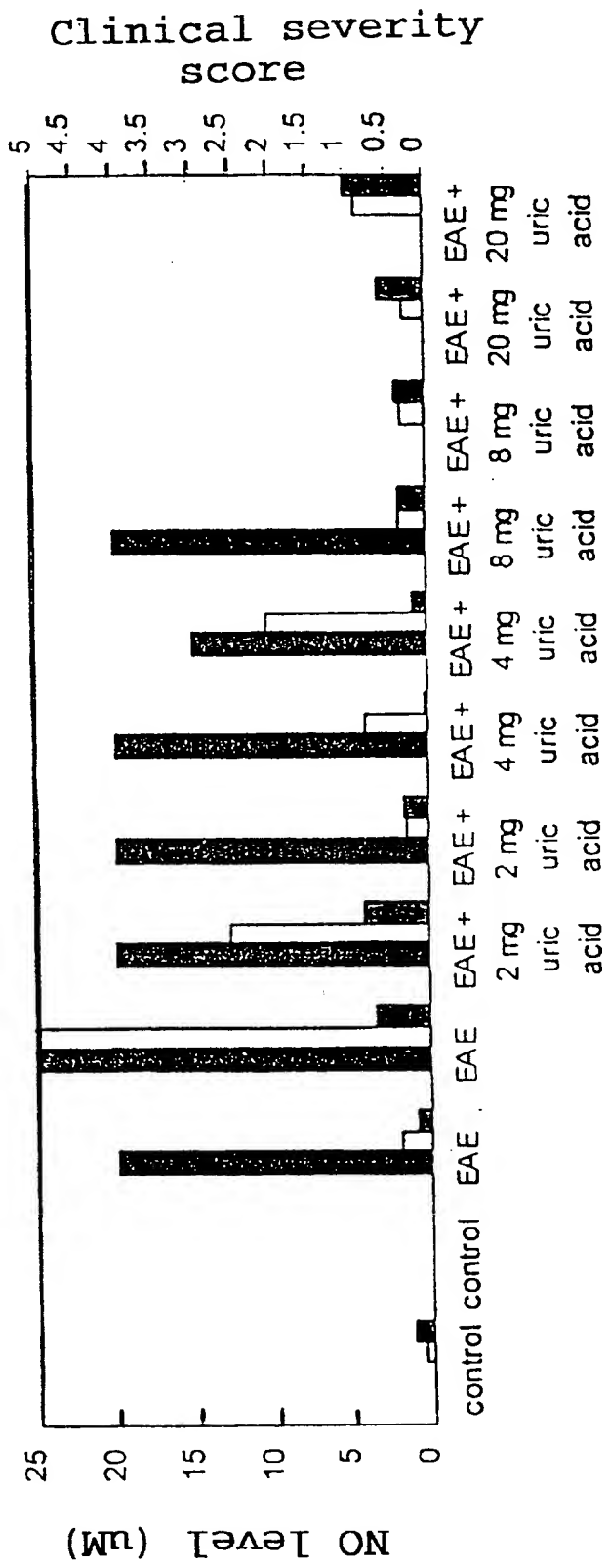


Fig. 14

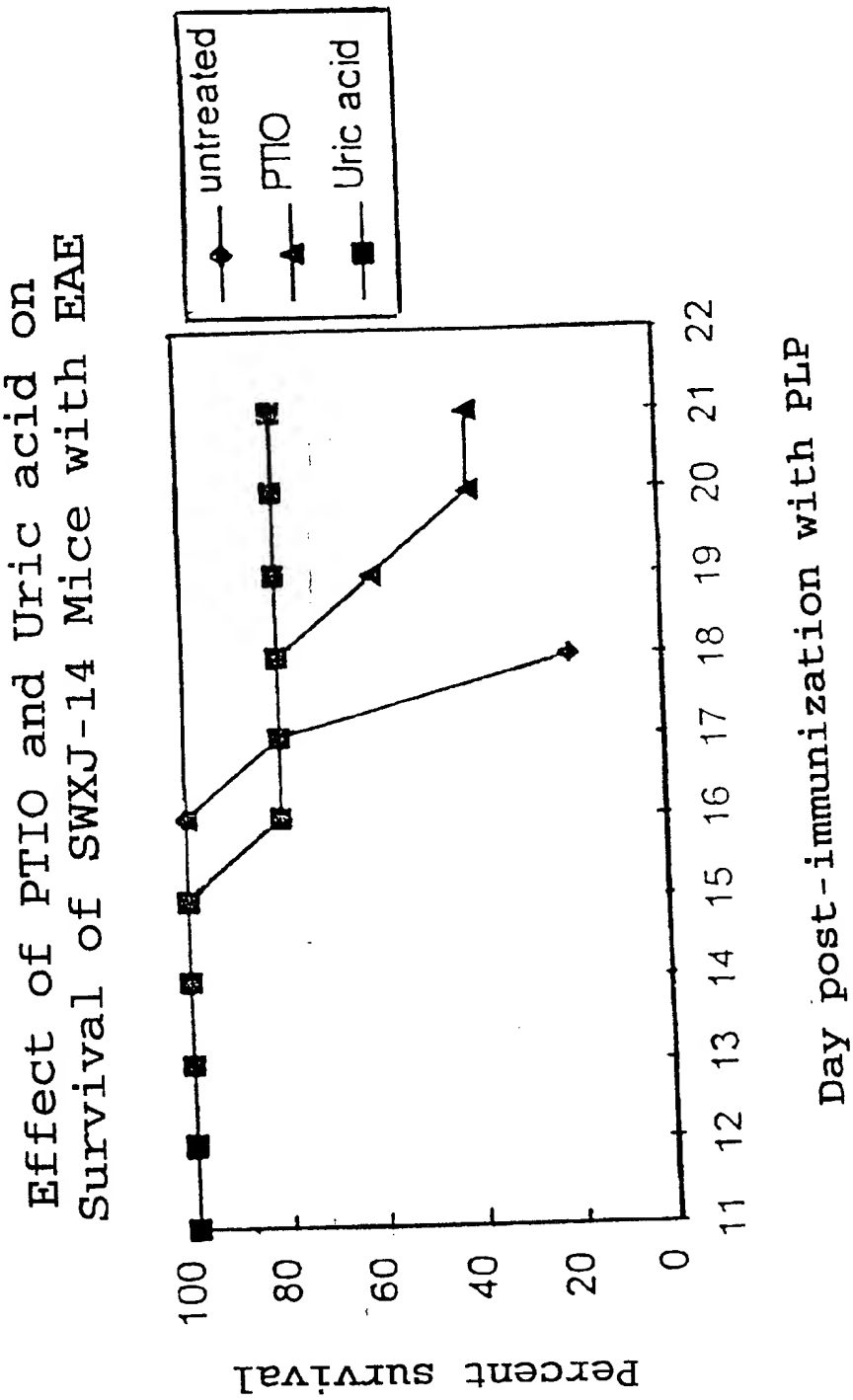
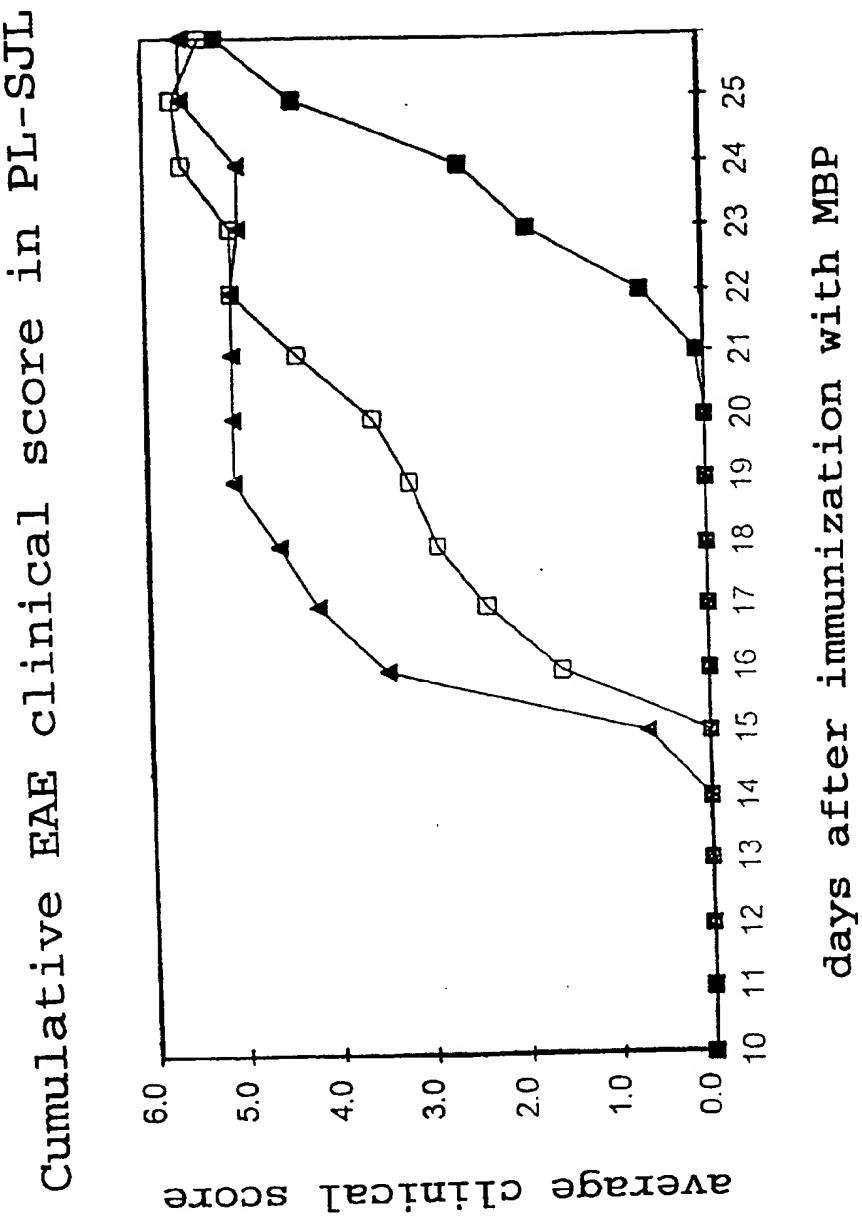

Effect of PTIO and Uric acid on
Survival of SWXJ-14 Mice with EAE
untreated
PTIO
Uric acid
Percent survival
100
80
60
40
20
0
11 12 13 14 15 16 17 18 19 20 21 22
Day post-immunization with PLP

Fig. 15


Cumulative EAE clinical score in PL-SJL
average clinical score
6.0
5.0
4.0
3.0
2.0
1.0
0.0
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
days after immunization with MBP

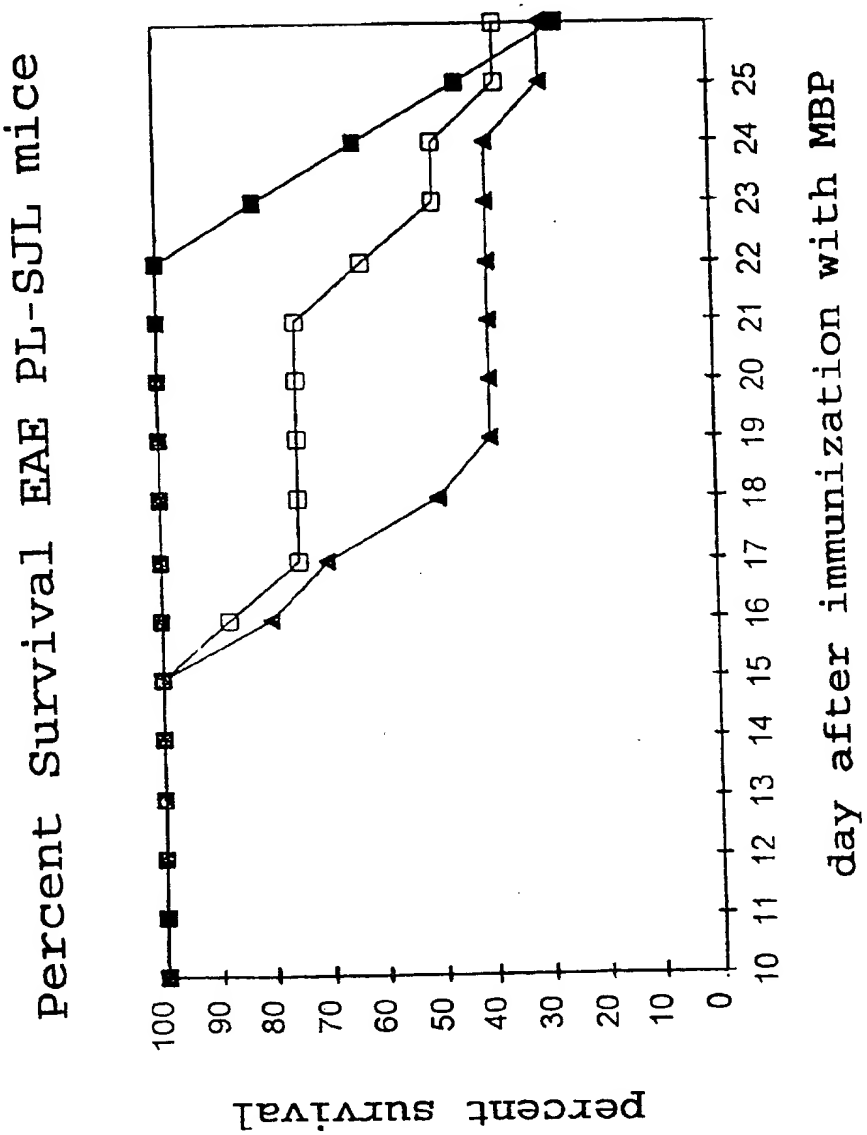

Percent Survival EAE PL-SJL mice
percent survival
100
90
80
70
60
50
40
30
20
10
0
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
day after immunization with MBP

Fig. 16

Fig. 17

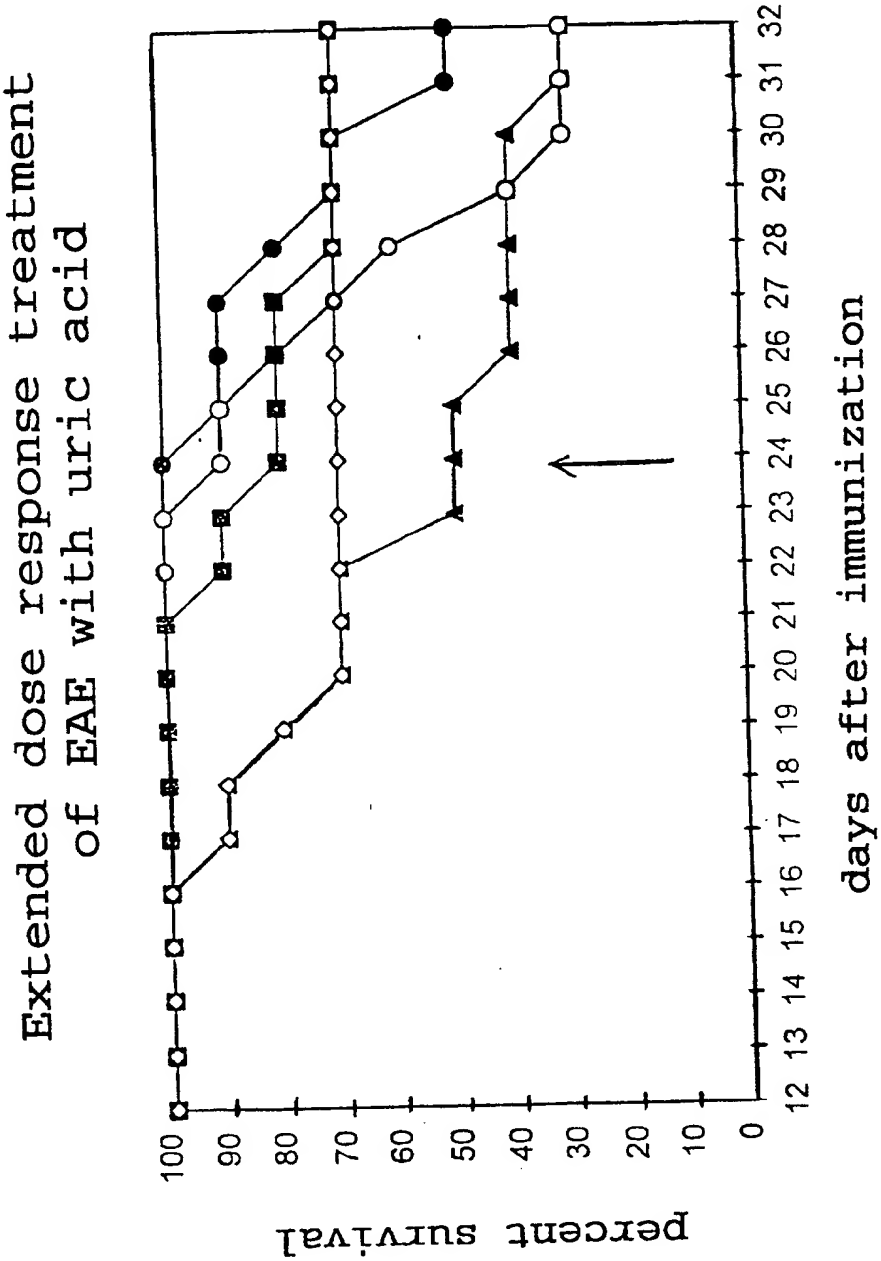

Extended dose response treatment
of EAE with uric acid
percent survival
100
90
80
70
60
50
40
30
20
10
0
12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
days after immunization

Fig. 18

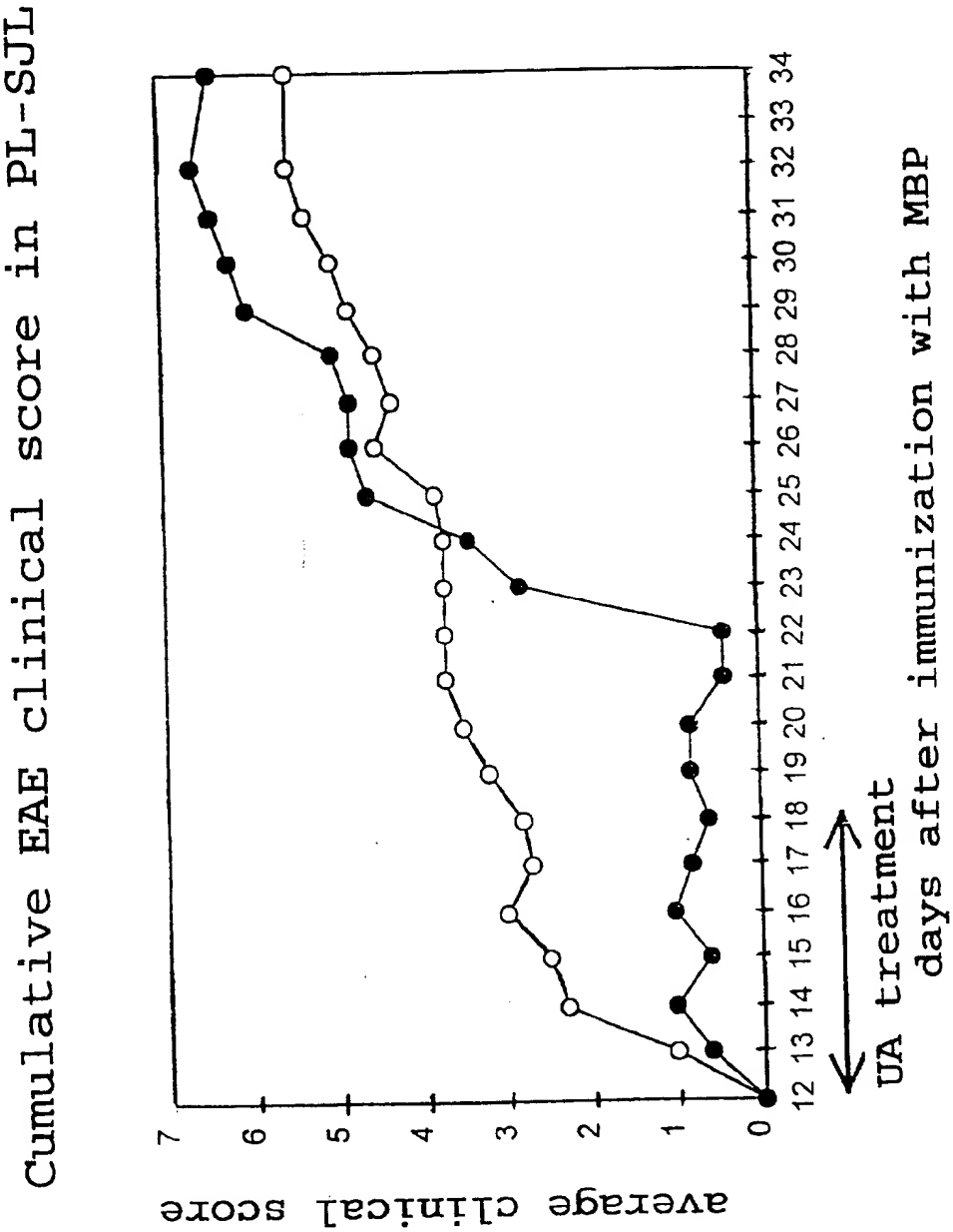

Cumulative EAE clinical score in PL-SJL
average clinical score
0
1
2
3
4
5
6
7
12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
days after immunization with MBP
UA treatment

Fig. 19

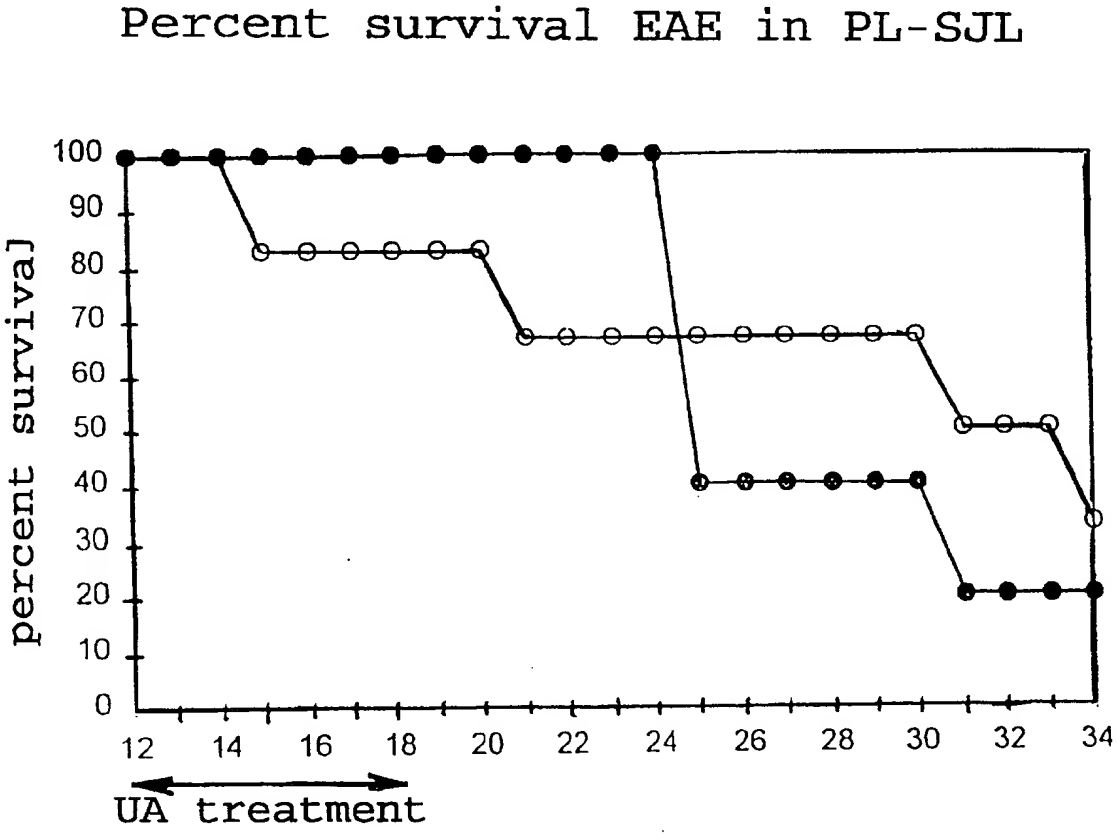

Percent survival EAE in PL-SJL
percent survival
100
90
80
70
60
50
40
30
20
10
0
12
14
16
18
20
22
24
26
28
30
32
34
UA treatment
days after immunization with MBP

Fig. 20

Cumulative EAE clinical score in PL-SJL

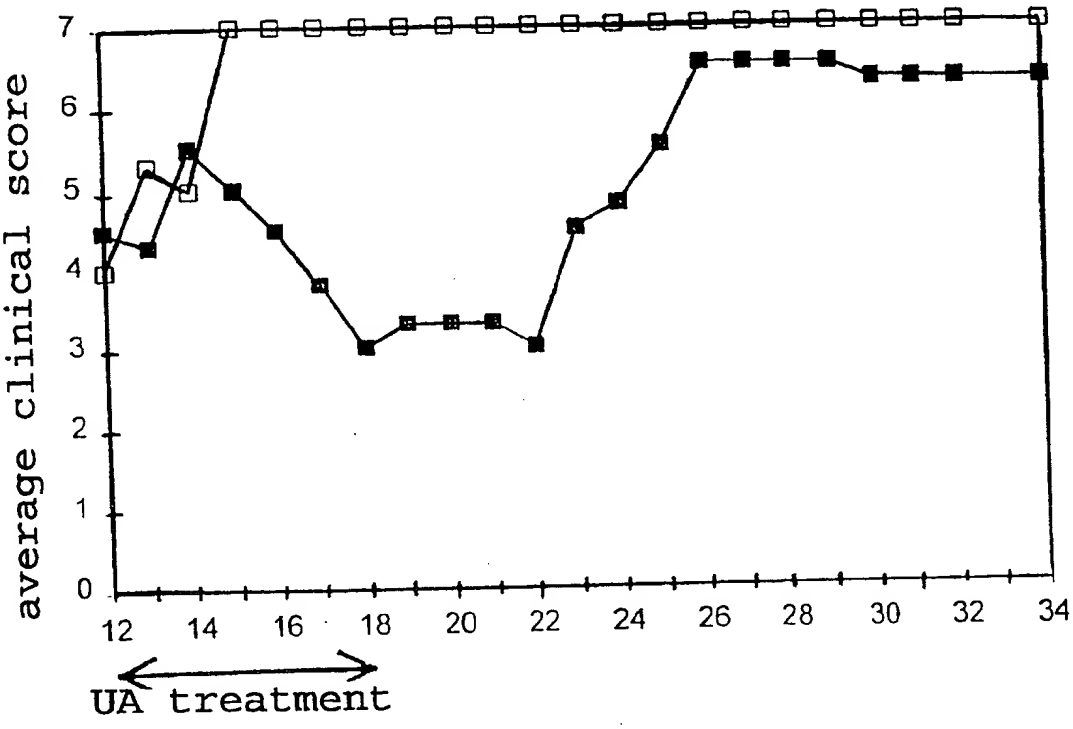
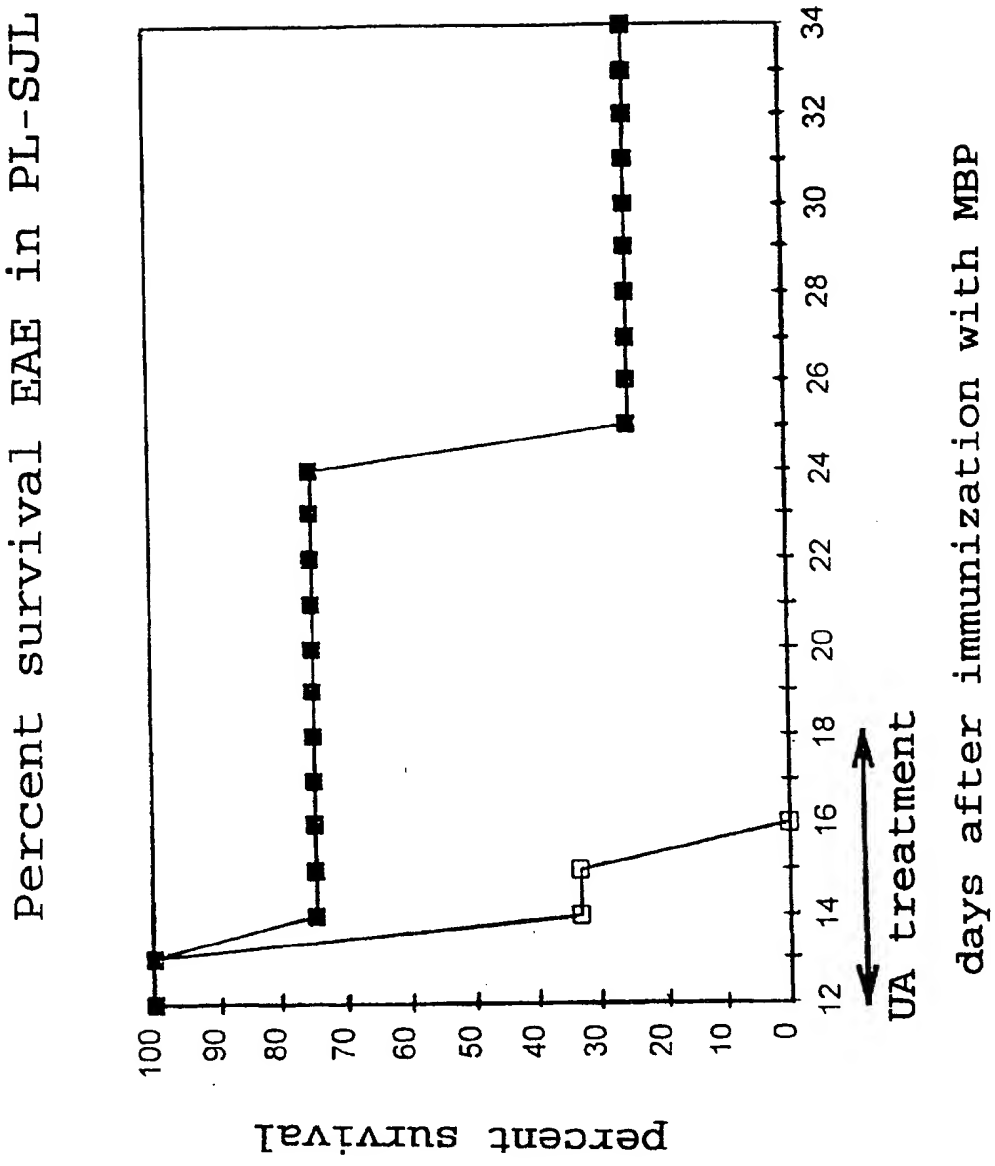


Fig. 21


Percent survival EAE in PL-SJL
percent survival
100
90
80
70
60
50
40
30
20
10
0
12
14
16
18
20
22
24
26
28
30
32
34
UA treatment
days after immunization with MBP

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/13547

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 43/00, 43/90 43/50
US CL : 514/183, 266, 386, 387

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/183, 266, 386, 387

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS dialog, medlin, ca, caplus,wpi
search terms:NO, peroxynitrite, scavenger?, central nervous system, diseases, inos

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CROSS, A. H. ET AL. AMINOGUANIDINE, AN INHIBITOR OF INDUCIBLE NITRIC OXIDE SYNTHASE, AMELIORATES EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS IN SJL MICE. J CLIN INVEST. 1994. VOL 93. PAGES 2684-2690, ENTIRE DOCUMENT.	1-23
Y	DAWSON, V. L. ET AL. NITRIC OXIDE NEUROTOXICITY. J CHEM. NEUROANAT. 1996. VOL 10. PAGES 179-190, ENTIRE DOCUMENT.	1-23
Y	HEALES, S. J. ET AL. TROLOX PROTECTS MITOCHONDRIAL COMPLEX IV FROM NITRIC OXIDE- MEDIATED DAMAGE IN ASTROCYTES. BRAIN RES. 1994. VOL 668. PAGES 243-245, ENTIRE DOCUMENT.	1-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

11 SEPTEMBER 1997

Date of mailing of the international search report

15 OCT 1997

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/13547

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IADECOLA, C. et al. Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. Am.J Physiol.1995. Vol. 268. pages R286-R292, entire document.	1-23
Y	KOOY, N. W. et al. Agonist-induced peroxynitrite production from endothelial cells. Arch.Biochem.Biophys.1994. Vol. 310. pages 352-359, entire document.	1-23